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*EFFECT OF ULTRAVIOLET IRRADIATION OF BACTERIOPHAGE T2 ON ENZYME SYNTHESIS IN HOST CELLS**

By Marie-Luise Dirksen, John S. Wiberg,† James F. Koerner, and John M. Buchanan

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Communicated by S. E. Luria, September 12, 1960

The infection of *Escherichia coli* by bacteriophage T2 results in the appearance of a number of novel enzyme activities which cannot be detected in the host cell prior to this event.[1–5] In addition, infection is followed by an increase in level of certain constituent enzyme activities of the bacterium.[2, 6, 7]

Studies of phage-induced enzyme production as a function of the time after infection have shown that after a lag period of 2 to 5 min the levels of these enzymes increase linearly up to 10 to 12 min after infection and thereafter tend to remain at a more or less constant value.

Flaks *et al.*[8] have shown that the concentration of deoxycytidylate hydroxymethylase measured 15 min after infection of *E. coli* with ultraviolet irradiated phage T2 remains essentially unaltered within certain doses of radiation while phage production decreases logarithmically under these conditions.

In this laboratory, a more detailed study has been made of the production of this enzyme as a function both of time after infection and of dose of ultraviolet light on the infecting phage. It has been shown that up to a certain level of radiation there is indeed little or no effect detectable in the kinetics of deoxycytidylate hydroxymethylase production up to 10 or 12 min after infection. Beyond this time, however, a rather striking divergence between the behavior of the irradiated samples and that of the unirradiated control becomes apparent. Instead of the normal cessation of enzyme formation, the samples infected with phage treated with ultraviolet light exhibit a continued linear increase up to 30 to 60 min after infection. The results of these experiments are presented below.

Methods and Materials.—Irradiation of phage T2: During irradiation phage T2Hr$^+$ were suspended in glucose-ammonium chloride medium[9] at a concentration of 4.7 to 5.5 $\times$ 10^{11} particles per ml. Three ml of the above phage suspension in quartz cuvettes were irradiated for the intervals indicated with stirring. The unfiltered output of a 4-watt General Electric germicidal lamp was used as the source of radiation. The survival of the phage was determined from plaque counts obtained by plating suitable dilutions of T2 suspension before and after irradiation. The plates were incubated in the dark to prevent photoreactivation.

Infection of E. coli with irradiated phage T2: *E. coli* B was grown in the glycerol-

casamino acid medium of Fraser and Jerrel[10] with vigorous aeration to a concentration of approximately 1×10^9 cells per ml. The culture was chilled to prevent further growth. Aliquots of 10 ml of bacterial culture were placed in chilled 500 ml Erlenmeyer flasks. Prior to the addition of phage each flask was placed on a shaker in a water bath at 37°C and preincubated for one min. Then 0.1 ml of phage T2 suspension was added (approximately 5×10^{11} infectious phage per ml before irradiation). Aliquots of 1 ml were removed from each incubation vessel at the times indicated and immediately frozen by pipetting them into liquid nitrogen. The frozen aliquots were stored at −20°C for 1 to 2 days. To release the enzymes the cells were disrupted in a Hughes press.[11] The extracts obtained in this manner were used without further purification for enzyme assays.

Determination of efficiency of adsorption of phage by E. coli: Just prior to the preincubation period an aliquot of the chilled *E. coli* culture was plated for colony count. Five min after infection 0.1 ml aliquots were removed and rapidly diluted into chilled nutrient broth, an aliquot of which was quickly removed and spread on agar for colony counts of viable *E. coli*. In the samples treated with either unirradiated or irradiated phage 3 to 4 per cent of the original *E. coli* input could be recovered as viable cells after adsorption of phage had been allowed to take place.

The maximum number of infected bacteria that liberate phage after infection with phage irradiated for one min was measured. It was found to be less than 1.5 per cent of that of a culture infected with untreated phage.

Enzyme assays: Deoxycytidylate hydroxymethylase was assayed by measurement of the incorporation of C^{14}-labeled formaldehyde into dHMP.[‡] The reaction mixture, similar to that described by Flaks and Cohen,[12] contained in 0.5 ml total volume: tris (hydroxymethyl) aminomethane buffer (acetate salt), pH 8.0, 25 μmoles; ethylenediamine tetraacetate, pH 8.0, 10 μmoles; dCMP, 2.1 μmoles; 2-mercaptoethanol, 10 μmoles; tetrahydrofolate,[13] 0.45 μmole; C^{14}-labeled formaldehyde, 1 μmole with a specific activity of 0.62 μcurie per μmole; enzyme extract containing the material from 4×10^7 cells. After incubation at 37° for 45 min, the reaction was terminated by chilling the vessels and adding 1 ml of a solution which was 0.2 *M* with respect to unlabeled formaldehyde and 1.85×10^{-5} *M* with respect to carrier dHMP. The enzyme was then inactivated by heating on a boiling water bath for 2 min. The reaction mixture was added to a Dowex 1-8x formate ion exchange column (3.3 mm in diameter × 50 mm in length; 200–400 mesh resin). After adsorption of the solution, the column was washed with 5 ml of water followed by 8 ml of 0.015 *M* ammonium formate buffer, pH 3.0. Elution of dHMP was accomplished with an additional 8 ml of the same buffer. The entire eluate containing the dHMP was dried on an aluminum planchet and the radioactivity was measured.

The assay for dCTPase has been previously described.[14] The assay for dTMP kinase was performed by incubation of enzyme and C^{14}-labeled dTMP under the conditions described by Lehman *et al.*[15] followed by separation of the radioactive dTTP by means of a Dowex-1-formate ion exchange column.

Results and Discussion.—Figure 1 represents a plot of the values of deoxycytidylate hydroxymethylase levels versus time after infection of *E. coli* with phage T2 irradiated with varying doses of ultraviolet light. Inspection of this graph reveals (*a*) that with the lower doses of ultraviolet light (curves B and C) there is very

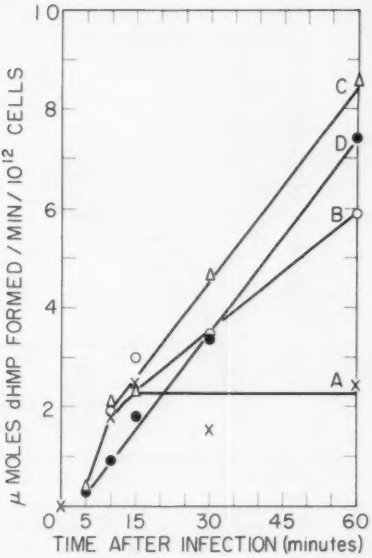

FIG. 1.—Deoxycytidylate hydroxymethylase activity as a function of time after infection of *E. coli* B with phage T2 irradiated with varying doses of ultraviolet light.
E. coli input, 8×10^8 cells per ml.
Phage input, 4.7×10^9 particles per ml.

Designation on graph	Time of irradiation (min)	Fraction of plaque forming phage after irradiation
X	0	1
○	0.5	1.2×10^{-3}
△	1	7.2×10^{-6}
●	1.5	2.8×10^{-7}

little, if any, change in the time of appearance of the enzyme and in the initial rate of its production as compared to that in cells infected with unirradiated phage (curve A); (*b*) that with all cells infected with irradiated phage a linear increase in enzyme concentration is observed after 15 min of infection, whereas in cells treated with unirradiated phage no change of enzyme level occurs after this time; (*c*) that the portion of the curves (A, B, and C) representing the enzyme levels beyond the first twelve min after infection show an increase in slope as the radiation dose to the phage is increased; (*d*) that at higher doses of ultraviolet light (curve D) the initial rate of production of the enzyme is slower than that in the control cells infected with unirradiated phage (curve A); however, the final value of enzyme activity in the cells infected with irradiated phage exceeds that of the control since no decline in the rate of enzyme production after 15 min occurs in the former case.

In Figure 2 are presented data of an experiment in which the hydroxymethylase synthesis by cells infected with unirradiated phage is compared with that by cells infected with phage irradiated for 1 min. The synthesis was followed over the duration of 4 hr. The results of this experiment confirm those shown in Figure 1. Again in the case of cells infected with unirradiated phage the level of enzyme reached a maximum at approximately 10 min and then remained constant for the duration of the experiment. In contrast, the cells infected with irradiated phage continued to produce enzyme at the initial rate for 30 min, after which time there was further enzyme formation but at a slower rate.

The most striking feature of these data comparing the effects of irradiated and unirradiated phage is the divergence of the pattern of enzyme synthesis beyond 10 min after infection. These results are consistent with the hypothesis that ultraviolet irradiation of phage causes a lesion in some system responsible for cessation of enzyme formation during normal infection.

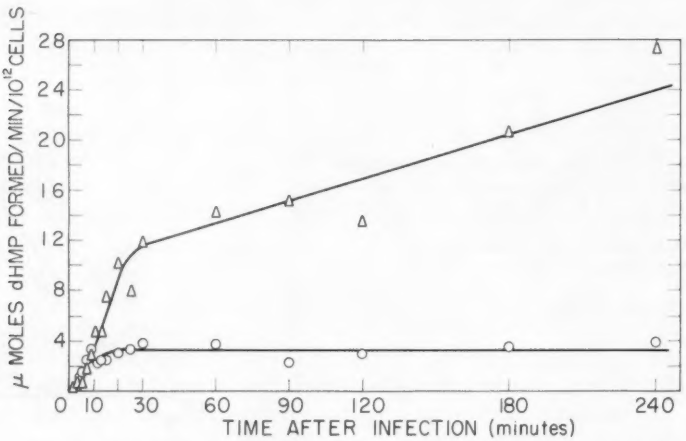

FIG. 2.—Deoxycytidylate hydroxymethylase activity as a function of time after infection of *E. coli* B with unirradiated and irradiated phage T2.

E. coli input, 1×10^9 cells/ml.
Phage input, 4.6×10^9 particles/ml.
Time of irradiation: 1 min.
Concentration of phage during irradiation: 4.6×10^{11} particles/ml.
Fraction of phage surviving irradiation: 8×10^{-6}.

The volumes of *E. coli* culture, phage suspension and incubation flask were 4 times those given under *Methods and Materials*.

○ unirradiated phage T2
△ irradiated phage T2

In the period before 10 min the rate of enzyme production is affected only when the infecting phage were exposed to the highest dose of ultraviolet light; under such circumstances the initial rate of enzyme formation is lower than that which occurs after infection with untreated phage. These facts are presented in favor of the argument that the system responsible for the initiation of enzyme synthesis, although radiation sensitive, is more resistant than the system responsible for arrest of the synthesis during the later period.

Preliminary investigations into the possible generality of this phenomenon for other phage-induced enzymes have been carried out by assaying for dCTPase,[2, 3] another enzyme detectable only after phage infection, and for dTMP kinase,[2, 6] an enzyme which is present in uninfected cells but which greatly increases after infection. In both these cases enzyme formation continues beyond the initial 10- to 12-min period whenever infection is produced by irradiated phage, as in the case of hydroxymethylase. With these two enzymes, however, the initial rate of enzyme formation is significantly decreased at a radiation level (1 min) which does not effect the initial rate of hydroxymethylase formation. Thus, it appears that the factor or factors responsible for the initial formation of dCTPase and of dTMP kinase are more sensitive to ultraviolet light than that for the induction of hydroxymethylase. These differences are illustrated in the case of dTMP kinase by the data shown in Figure 3.

The cause for prolonged enzyme synthesis in the host cells upon infection with

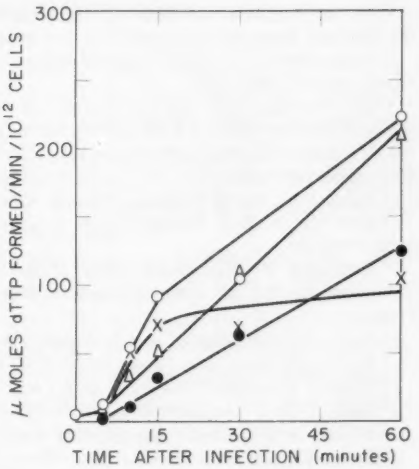

FIG. 3.—Thymidylate kinase activity as a function of time after infection of *E. coli* B with phage T2 irradiated at varying levels with ultraviolet light. The assays were performed on the same extracts as those for deoxycytidylate hydroxymethylase presented in Fig. 1.

Designation on graph	Time of irradiation of phage (min)
X	0
○	0.5
△	1
●	1.5

irradiated phage has not been determined. In searching for an explanation for this phenomenon two major questions may be posed. (1) Does infection of *E. coli* by unirradiated T2 cause the formation in the host cell of a chemical entity which has the function of controlling the levels of induced enzymes directly? Or (2) does the appearance or accumulation in sufficient quantity of any one of the components required for phage formation play an indirect regulatory role in enzyme synthesis? Among substances which might be involved in such an indirect role, DNA, phage structural proteins, or phage lysozyme may be mentioned. New phage DNA first appears 7 min after normal infection[16, 17] but fails to do so when infection is caused by irradiated phage.[8, 17] Structural proteins[18, 19] and lysozyme can be detected at about 10 min after infection. Such an indirect control could be exerted by the physical presence of these entities. Alternatively, the initiation of the synthesis of the structural proteins and lysozyme during infection by the unirradiated phage may result in some manner in the diversion of metabolic precursors from the synthesis of the early enzymes to that of these other proteins.

The fact that infection with irradiated phage results in a greater level of these enzymes in comparison to infection with unirradiated phage is of practical value in their isolation.

Summary.—The kinetics of formation of deoxycytidylate hydroxymethylase, an enzyme induced in *E. coli* by infection with phage T2, have been studied. Under the usual conditions of infection, enzyme formation takes place during the time interval of 3 to 12 min after infection, after which time the enzyme level remains approximately constant. If the cells are infected with phage which have been irradiated with ultraviolet light at an appropriate dose, the kinetics of enzyme formation during the first ten minutes are similar to those observed upon infection with unirradiated phage. However, enzyme formation continues for a longer period of time after infection with the irradiated phage. This effect has also been noted with two other phage-induced enzymes, deoxycytidine triphosphatase and thymidylate kinase. Possible implications of this finding are discussed.

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† Postdoctoral fellow of the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service (1959–1960).

‡ The following abbreviations are used: dCMP, dCTP, the mono- and triphosphate, respectively, of deoxycytidine; dTMP, dTTP, the mono- and triphosphate, respectively, of thymidine; dHMP, deoxy-5-hydroxymethylcytidylate; dCTPase, deoxycytidine triphosphatase; DNA, deoxyribonucleic acid.

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*A POSSIBLE ROLE FOR ARGININE IN ENZYME MECHANISMS**

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Communicated by David Rittenberg, September 20, 1960

It has been proposed that a histidine residue plays an essential part in the catalytic activities of a number of enzymes.[1] The evidence cited in support of this hypothesis is extremely suggestive but, on the whole, indirect. A major part can be summarized as follows:

(*a*) The pH-activity curves of a large number of enzymes suggest the participation of a group having a pK_a in the region of 6.5. The imidazolium ion dissociates in this region; the ionizable functional groups of other amino acids normally do not.

(*b*) Certain monoacylated enzymes, such as acetyl chymotrypsin, are labile, losing their acyl group rapidly in neutral and slightly basic solutions. Acylated imidazole compounds behave in a similar manner.

(*c*) Imidazole derivatives are capable of catalyzing the hydrolysis of nitrophenyl esters and, when suitably designed, will catalyze the hydrolysis of other esters and amides.

(*d*) Data derived from photo-oxidation studies have been interpreted as indicating a relationship between the destruction of histidine residues and the loss of activity of such enzymes as lysozyme, maltase, chymotrypsin, and ribonuclease. In our opinion, the data do not necessarily support this relationship, especially, for example, in the case of lysozyme where 30 per cent of the activity was retained even after all of its histidine was oxidized.[2]

To the above should also be added the more direct evidence derived from the elegant experiments of Barnard and Stein, which appears to demonstrate the essential role of histidine in the activity of ribonuclease.[3]

The evidence cited lends support to the hypothesis that histidine participates in at least some enzyme mechanisms. Suggestions as to the manner in which it plays a role are numerous[1] and depend upon the enzymatic reaction under consideration. This paper will deal mainly with enzymes that were found to possess an unusually reactive serine at their active site. Some examples are trypsin, chymotrypsin, phosphoglucomutase, and a number of esterases. For these enzymes it has been suggested that the enhanced nucleophilic activity of the serine residue is the result of an interaction with the imidazole nucleus of a neighboring histidine residue.[4, 5] Other hypotheses, which do not invoke an interaction with histidine have also been put forth to explain the unusual reactivity of the serine residue. In these it is proposed that the hydroxyl group of the serine is involved in an oxazolone or more complex, reactive linkage.[6, 7]

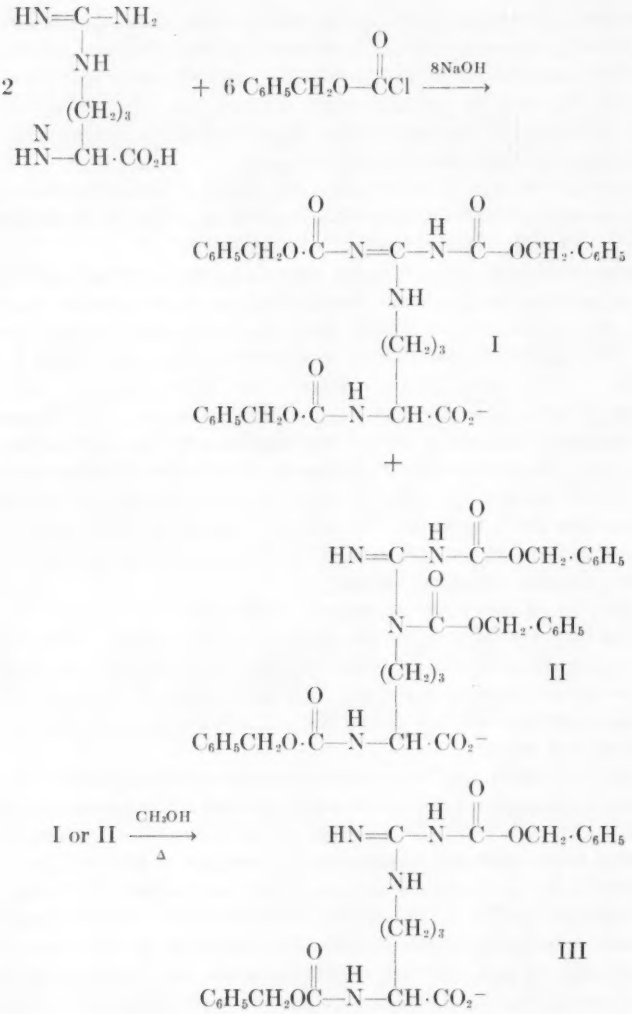
It is the purpose of this paper to suggest still another alternative, one that is based upon the fact that the guanido group of an arginine residue, under conditions that exist in physiological systems, can possess chemical and physicochemical properties not unlike those of histidine. The mechanism of catalysis herein proposed postulates an interaction between the guanido group of an arginine residue and the hydroxyl of serine.

In 1957, Zervas, Winitz, and Greenstein[8] reported a reaction between L-arginine and an excess of carbobenzoxy chloride which yielded a compound that analyzed as the sodium salt of N^{α}, N^{ω}, N^{ω}-tricarbobenzoxy L-arginine. Recrystallization of this material from methanol resulted in the recovery of only 50% of the total product. Present in the mother liquor was a new compound which, after acidification, was identified as N^{α}, N^{ω}-dicarbobenzoxy-L-arginine. It was apparent that the crude product of the acylation reaction was composed of two triacylated derivatives, a stable one and one with an alcohol-labile carbobenzoxy group. The latter isomer was called the "alcohol susceptible" derivative. The reactions are pictured on page 1432.

The di-acylated compound (III) could be re-carbobenzoxylated but now the resulting tri-carbobenzoxy L-arginine consisted mainly of the "alcohol-susceptible" isomer.

Zervas and his collaborators did not attempt to define the term "alcohol susceptibility" in a chemical sense. It is apparent, however, that acylation of an acylated guanido group can produce a product which, like acylated imidazole, deacylates under mild conditions, probably by a transfer reaction with methanol. More recently Greenhalgh and Bannard reported[9] that triacetyl guanidine is alcohol-labile, undergoing stepwise deacetylation to guanidine acetate.

N^{α}, N^{ω}-diacyl arginine derivatives had been studied earlier by Felix and Dirr[10]



and by Zervas and Bergmann,[11] who observed that acylation of the guanido group did not completely suppress its basic properties. This was confirmed by Gish and Carpenter[12] who reported the pK_a of the acylated guanido groups of N^{α}, N^{ω}-di-p-nitrobenzyloxycarbonyl L-arginine, its methyl ester, and N^{ω}-p-nitrobenzyloxycarbonyl L-arginine to be 6.45, 5.35, and 8.80, respectively. Titration of N^{α}, N^{ω}-dicarbobenzoxy L-arginine in 1:1 dioxane-water in our laboratory yielded pK_a values of 4.40 and 6.80 for the carboxyl and guanido groups, respectively. Thus, acylation suppresses the basicity of the guanido group of arginine, resulting in a pK_a in the region of neutrality.

The acylated guanido group therefore has two interesting properties: (1) It

can be acylated to yield a derivative that deacylates under mild conditions; (2) It is less basic than the free guanido group, having a pK near neutrality. Both properties are possessed by the imidazole nucleus of histidine.

An examination of the ultraviolet spectra of the carbobenzoxylated derivatives of arginine was undertaken in this laboratory. The compounds were studied in methanol solution where, at room temperature, the "alcohol-susceptible" derivative decomposed at a rate slow enough for its spectrum to be measured by a recording spectrophotometer. The results are shown in Figures 1 and 2.

Figure 1 shows the spectra of the two tri-acylated isomers and di-carbobenzoxy

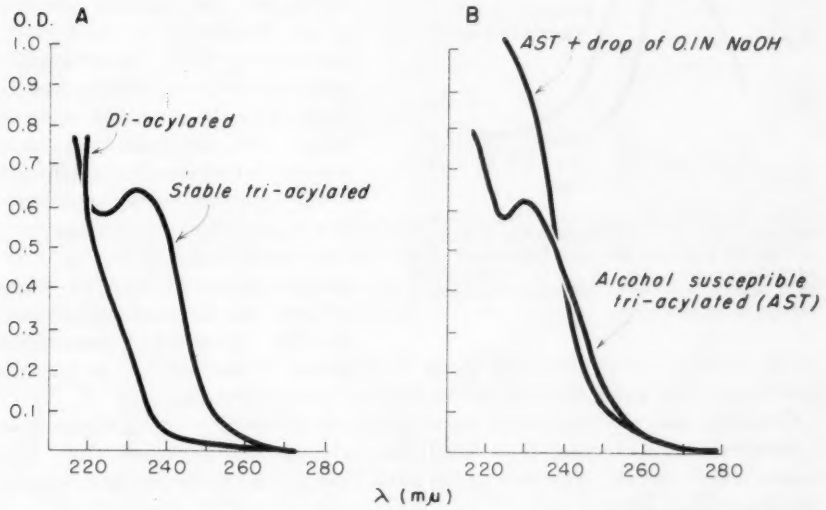


FIG. 1.—(*a*) Ultraviolet spectra of N^{α},N^{ω}-dicarbobenzoxy L-arginine and sodium salt of stable $N^{\alpha},N^{\omega},N^{\omega}$-tricarbobenzoxy L-arginine in methanol; concentration, 3.3×10^{-5} M.
(*b*) Ultraviolet spectrum of sodium salt of "alcohol susceptible" $N^{\alpha},N^{\omega},N^{\omega}$-tricarbobenzoxy L-arginine (AST) in methanol, concentration 3.3×10^{-5} M, and spectrum of same solution 3 mins after one drop of 0.1 N NaOH was added.

L-arginine. A shoulder, present in the spectra of both tri-substituted derivatives, does not occur in the spectrum of N^{α}, N^{ω}-dicarbobenzoxy L-arginine. The addition of a drop of dilute base to the methanol solution of the "alcohol susceptible" derivative catalyzes the de-acylation reaction and causes an immediate disappearance of the shoulder. The spectrum now resembles that of the di-acylated derivative, although shifted toward the higher wavelengths because of the presence of the product of the acyl transfer reaction.

Figure 2 shows the difference spectrum of the labile tri-acylated derivative read against dicarbobenzoxy L-arginine. A peak which is no longer present after de-acylation occurs at 234 mμ (Fig. 2). The position of this peak could indicate the presence of a conjugated double bond system, a structure that occurs in compound I The spectral data suggest, therefore, that compound I is the "alcohol susceptible" isomer.

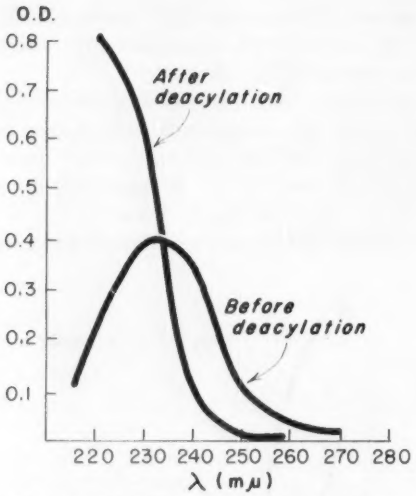
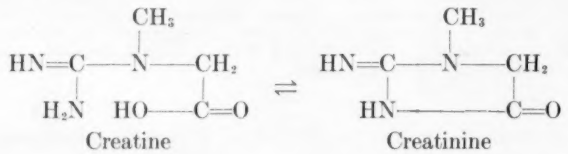


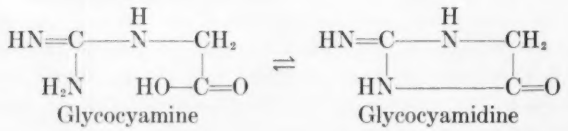
FIG. 2.—Difference spectra of "alcohol susceptible" Nα,Nω,Nω-tricarbobenzoxy L-arginine (3.3×10^{-5} M) in methanol before and after addition of 1 drop of 0.1 N NaOH—read against an equimolar solution of Nα,Nω-dicarbobenzoxy L-arginine in methanol.

The imidazole nucleus, when acetylated, shows an absorption maximum at 245 mμ.[13] However, when carbobenzoxy is the acyl group, as in 1,N-dicarbobenzoxy L-histidine, the wavelength of the maximum drops to 238 mμ[14] rather close to the 234 mμ maximum in Figure 2, thus demonstrating another physicochemical similarity between imidazole and the acylated guanido group. It should be mentioned, furthermore, that decarbobenzyloxylation of 1,N-dicarbobenzoxy L-histidine at the imidazole nitrogen occurs very slowly at room temperature in methanol but is complete within a few minutes in the presence of a catalytic quantity of base.[14]

The preceding data show that the guanido group of arginine, when acylated, has chemical and physicochemical properties remarkably similar to those of the imidazole group of histidine. It remains now to indicate how the guanido group can exist in the acylated form in proteins.

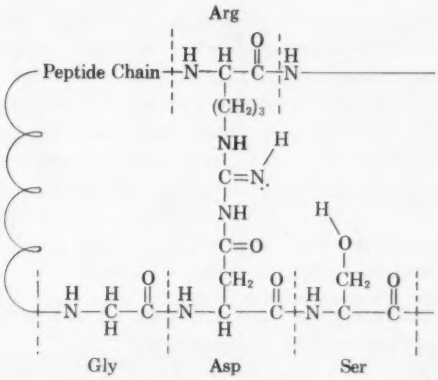
Creatinine and glycocyamidine are examples of naturally occurring compounds in which the guanido group is acylated, the acylation being intramolecular. The former is derived from creatine and the latter from glycocyamine by the reversible reactions shown below.



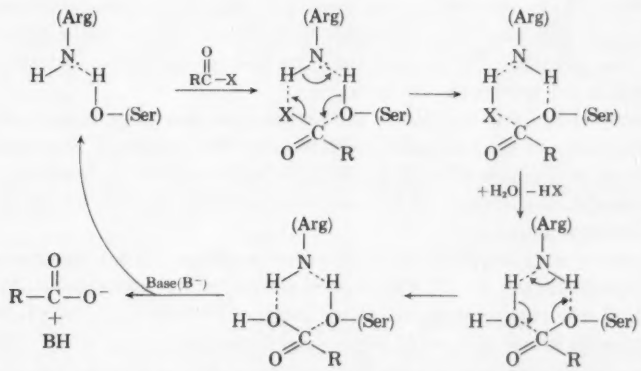


The relative concentrations of the compounds at equilibrium are determined by the pH of the solution. In both interconversions, equimolar concentrations are present at pH 7.[15, 16] Precedent therefore exists for the formation of acylated guanido groups under what might be termed physiological conditions. We suggest that the steric and energy requirements necessary for a similar intramolecular acylation of the guanido group of an arginine residue might be fulfilled in certain enzymes.

In the case of those enzymes containing the amino acid sequence -Gly-Asp-Ser-Gly- at the active center, we propose that the acylating group might be the carboxyl group of the aspartic acid residue. The type of structure which would result from this interaction is shown below:



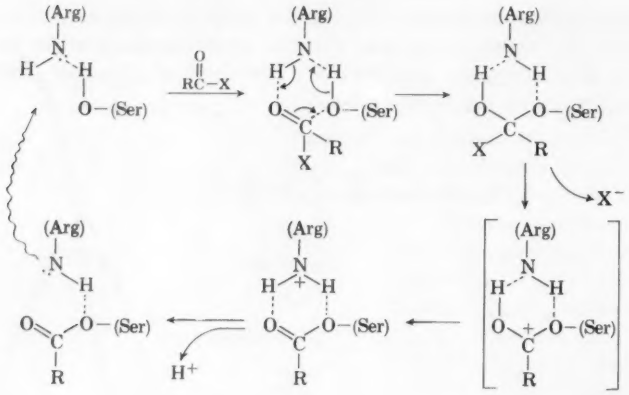
When a three dimensional model of this system is constructed, the imino group of arginine is found to be close enough to the hydroxyl of serine to form a hydrogen bond. It is possible, therefore, to suggest the following mechanism for the hydrolysis of a substrate $RC(=O)—X$.



(It is assumed that the hydroxyl group of serine is acylated during the enzymic process. An analogous mechanism can be proposed for an attack by the imide nitrogen.)

The above mechanism is essentially a concerted acid-base catalysis similar to that first described by Swain.[17] The involvement of a six-membered cyclic intermediate should be noted.

An alternative mechanism analogous to the one proposed by Cunningham[4] for the histidine-serine interaction can also be proposed. In this mechanism an acylated guanido group plays the role suggested for histidine.



Subsequent deacylation can be base catalyzed or may involve a nucleophilic attack by nitrogen, as suggested by Cunningham.[4]

Discussion.—Attributes of the hypothesis: (*a*) The hypothesis can explain the conversion of inactive zymogen to active enzyme. According to the findings of Neurath *et al.*,[18] changes in the secondary and tertiary structure occur during the activation of chymotrypsinogen and trypsinogen. We suggest that, as a result of these structural changes, a guanido group of an arginine residue is brought to a position in space that favors a creatine-creatinine type interaction with the carboxyl group of the aspartic acid residue situated at the active site of the enzyme.

(*b*) The hypothesis can explain the finding of Van Vunakis and Herriot[19] that α-chymotrypsin has one more titratable group than chymotrypsinogen, with a midpoint near pH 6.8. We suggest that the new group is the acylated guanido group, which is not present in the zymogen.

(*c*) Loss of enzymatic activity in the presence of urea or guanidine can be the result of disruption of the hydrogen bonds required for the proper placement of the guanido group of arginine relative to the carboxyl group of the acylating residue. Guanidine might, in addition, act by competing with the guanido group of arginine in the acylation reaction.

(*d*) Chymotrypsin, trypsin, and many other enzymes exhibit maximal activity at a pH of approximately 8. The increase of activity as the maximum is approached from the acid side can be explained if we assume that the uncharged form of the acylated guanido group (pK $\sim$ 6) is required for enzymatic activity. The fall in activity on the alkaline side can be the result of a shift in equilibrium toward deacylation of the guanido group, just as the creatine-creatinine reaction is displaced toward creatine at alkaline pH.[15, 16]

(*e*) Finally, the reaction mechanism is made attractive by the participation of a six-membered cyclic intermediate. This favorable type of structure is not obtainable with histidine because of the restrictions imposed by the rigid planar structure of the imidazole nucleus (cf. ref. 5).

The limitations of the hypothesis delineated in this paper are, to be sure, numerous. First of all, no evidence exists, as yet, for the occurrence of intramolecular acylation of guanido groups in proteins. Perhaps a search should be made among

proteins having "masked" carboxyl groups that become titratable in the presence of urea or guanidine.[20]

Secondly, there is a paucity of information about the chemical properties of arginine derivatives in which the guanido group is acylated. It would be especially important to study model peptides of this kind. We have attempted to determine whether the acylated guanidine compounds available to us could catalyze the hydrolysis of p-nitrophenylacetate. Negative results were obtained with N^{α}, N^{ω}-dicarbobenzoxy L-arginine, creatinine, and glycocyamidine when tested alone or in the presence of ethanol. These results however, do not preclude the possibility that this class of compound can participate in a multifunctional catalytic process.

Summary.—(*a*) Evidence has been presented for the possible existence in proteins of a functional group, other than imidazole, having a pK_a near neutrality—the acylated guanido group derived from arginine.

(*b*) It has been shown that the acylated guanido group possesses certain physicochemical and chemical properties similar to properties that have been cited as supporting the participation of the imidazole nucleus of histidine in enzymatic mechanisms.

(*c*) Mechanisms have been presented for the participation of the acylated guanido group in catalytic processes. These mechanisms are in accordance with experimental findings in the literature.

The author wishes to acknowledge the excellent technical assistance of Mr. Nicholas Kokowsky.

* This investigation was aided in part by a contract with the Office of Naval Research.

[1] For a complete discussion of the possible role of histidine in enzyme mechanisms, see the chapters by D. E. Koshland and F. H. Westheimer in *The Enzymes*, ed. P. D. Boyer, H. Lardy, and K. Myrback, (New York: Academic Press, Inc., 1959), Vol. 1, 2nd edition.

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*THE BIOGENESIS OF THE MOLD TROPOLONES**

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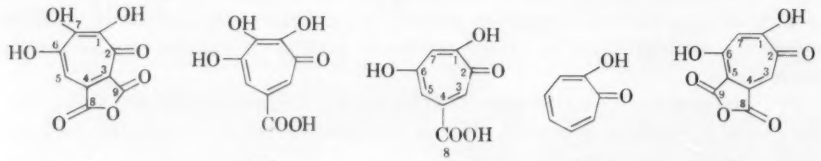
Communicated by Carl Niemann, September 19, 1960

The discovery of the mold metabolites puberulonic acid (I) and puberulic acid (II)[1] and stipitatic acid (III)[2] presented a difficult structural problem to organic chemistry until, in 1945, Dewar[3] made the ingenious proposal of the existence of tropolone (IV) as a new aromatic system. Dewar[3] correctly assigned the structure III to stipitatic acid. The work of Todd and his collaborators[4] led to I for puberulic acid. Aulin-Erdtman[5, 6] and Todd *et al.*[7] showed puberulonic acid to be II. Recently, a fourth mold tropolone, stipitatonic acid, has been characterized[8–10] as V.



I II III IV V

No less perplexing than the structural problem is the question of the biogenesis of this group of natural substances. Several proposals have been put forward.[11–15] The currently popular schemes envision tropolone biogenesis not to be the result of any known mode of sugar or acetate metabolism[14] but postulate, for example, the cyclization of an octulonic acid phosphate derivative.[15]

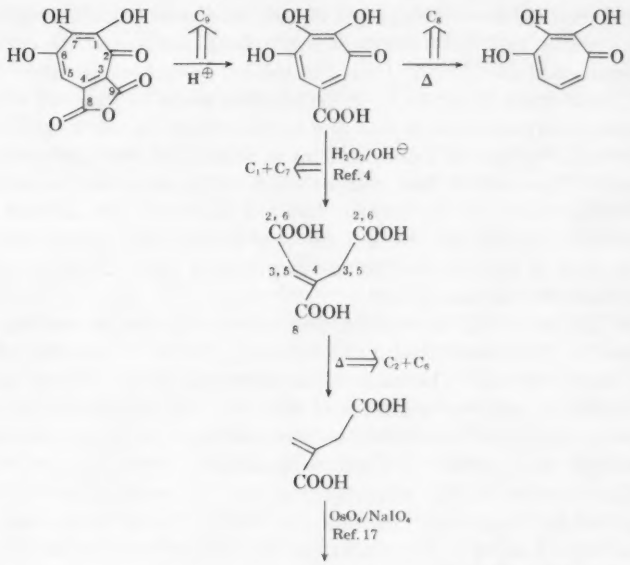
It is the purpose of this paper to present the results of a study on the incorporation of certain labeled substrates into puberulic acid and puberulonic acid by *Penicillium aurantio-virens*, and on the basis of these results to propose that the mold tropolones arise via a direct pathway from simple one and two carbon precursors. Moreover, we propose that they belong to the same general biosynthetic realm as such substances as orsellinic acid and cyclopaldic acid.§

Experimental.—To a standard Czapek-Dox nutrient medium was added a small amount of an appropriately labeled substrate. The medium was then innoculated with *Penicillium aurantio-virens* Biourge (77) CBS R2138.[1] Growth proceeded for 4–6 weeks, at which time puberulic acid and puberulonic acid were isolated and separated by the method of Barger and Dorrer.[16]

The acids were degraded by the following sequence, each step allowing the determination of the activities of certain carbon atoms as indicated.

The radioactivities of the samples were determined by combustion to carbon dioxide and counting in an ion chamber by means of a vibrating-reed electrometer. The results obtained are given in Table 1 and are expressed as the percentage activity in the indicated carbon atoms relative to puberulonic acid as 100 per cent.

The phenylenediamine derivative[18] and pyridine complex of puberulonic acid[18] and the diacetyl derivative of puberulic acid[2] were prepared and their activities compared with those of the parent compounds to insure that the observed activities



of puberulonic acid and puberulic acid were not inaccurate by reason of a highly active impurity. This precaution was taken in the degradation of tropolones derived from each substrate. In all cases, the activities of the derivatives were within 3 per cent of those of the parent compounds.

TABLE 1

PRECURSOR

Per cent activity in	1-C^{14} acetate	2-C^{14} acetate	1-C^{14} glucose	C^{14} formate
$C_1 + C_7$	...	24 ± 4	36 ± 4	100
$C_2 + C_6$	76 ± 12	...	...	...
$C_3 + C_5$	...	54 ± 4	31 ± 4	...
C_4	24 ± 14	...	...	...
C_8	...	22 ± 5	20 ± 4	...
C_9	...	...	13 ± 4	...

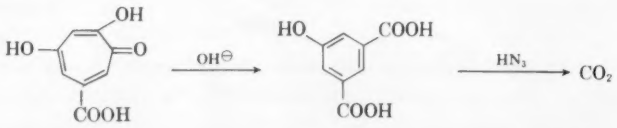
The incorporation of bicarbonate was also studied. It was very much less efficiently utilized than any of the other substrates investigated. Ten per cent of the activity was found in C_9. Further degradations have not been completed, but the very low level of incorporation suggests that carbon at the oxidation level of carbon dioxide is not a specific precursor of any of the carbon atoms of the mold tropolones.

Discussion.—The intermediacy of puberulic acid in the degradative sequence presents certain potential difficulties in the interpretation of the experimental results. Thus puberulic acid is, by virtue of enolization, a symmetrical molecule which may render ambiguous any distinctions between the origin of C_1 and C_7, between C_2 and C_6, and between C_3 and C_5. In the case of C_2, C_6 and C_3, C_5 this

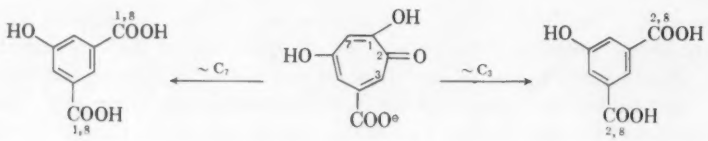
ambiguity is resolved as both members of each pair have a similar origin.

From the results with 2-C^{14} acetate it can be seen that four of the carbon atoms in puberulonic acid are derived from the methyl carbon of acetate. These are C_3, C_5, C_8, and either C_1 or C_7. An assumption made in this and other subsequent similar interpretations is that the carbon atoms in the tropolone derived from a particular carbon of a precursor are labeled to at least approximately the same extent. Thus the fact that, with 2-C^{14} acetate as precursor, 54 per cent of the activity is found in $C_3 + C_5$ implies that *both* C_3 and C_5 are derived from the methyl carbon of acetate and not that only one of these two carbons is so derived, with the activity in this position being twice that of other carbons (such as C_8) also derived from the acetate methyl.

Bentley[14] has previously described a degradation of stipitatic acid derived from 1-C^{14} acetate in which he concluded that C_4 and C_6 but *not* C_2 are derived from the acetate carboxyl carbon. The basis of the latter conclusion was the assumption that the base-catalyzed rearrangement of stipitatic acid would extrude, to a significant extent, both C_1 and C_2 into the new carboxyl carbon of the 5-hydroxybenzene-1,3-dicarboxylic acid product. When stipitatic acid biosynthesized from 1-C^{14} acetate was subjected to this rearrangement and the isophthalic acid derivative decarboxylated by the Schmidt reaction, the carbon dioxide so obtained had only a small activity—leading to the conclusion that the carboxyl carbon of acetate is not incorporated into C_2 of the tropolone skeleton.



We do not believe the assumption made in this deduction is valid. Stipitatic acid is not a symmetrical molecule, and the migration of either C_3 or C_7 will proceed along pathways of different energies. This will almost surely lead to a predominance of rearrangement in one of the two possible senses. Under the highly alkaline conditions of the rearrangement, the C_8 carboxyl group will be ionized. There is evidence[19] in the case of the benzilic acid rearrangement—a reaction analogous to the ring contraction of tropolones—that the site of attack by base controls the direction of rearrangement. In the case of stipitatic acid, therefore, attack of base at C_2 followed by migration of C_3 will be hindered by the presence of the carboxylate anion relative to attack of base at C_1 followed by migration of C_7. This will result in the preferential (not necessarily exclusive) occurrence of C_1 of stipitatic acid as the new carboxyl carbon in the 5-hydroxybenzene-1,3-dicarboxylic acid. Bentley's degradation does not, therefore, preclude the possibility that C_2 of the tropolone skeleton can orginate in the carboxyl carbon of acetate.

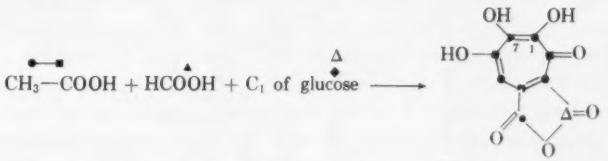


In fact, our results with 1-C^{14} acetate are most convincingly interpreted as indicating that C_2, C_4, and C_5 of the tropolone nucleus are derived from the acetate carboxyl carbon.

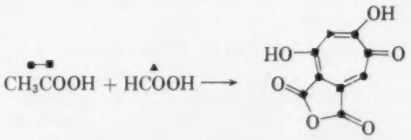
The results with C^{14} formate show that either C_1 or C_7 of puberulonic acid can be supplied by formate.

The experiments with 1-C^{14} glucose suggest the occurrence of six carbons in puberulonic acid, which can be derived from C_1 of glucose. These are: C_1, C_3, C_5, C_7, C_8, and C_9. We interpret this result as indicating that the glucose is first dissimilated to smaller fragments before its incorporation into the mold tropolones, and that the results with 1-C^{14} glucose are, in the main, the sum of those with 2-C^{14} acetate and C^{14} formate. The unusual exception is C_9 of puberulonic acid, which is not derived from either of the two acetate carbons nor from formate but is supplied by C_1 of glucose. The simplest, though by no means unique, explanation of this result is that C_9 is derived from a one-carbon pool, which can, in turn, be derived from C-1 of glucose, but that this particular one-carbon pool is not in significant metabolic contact with added sodium formate in *P. aurantio-virens*.

These results lead to the following proposal for the origin of the various carbon atoms of puberulonic acid:



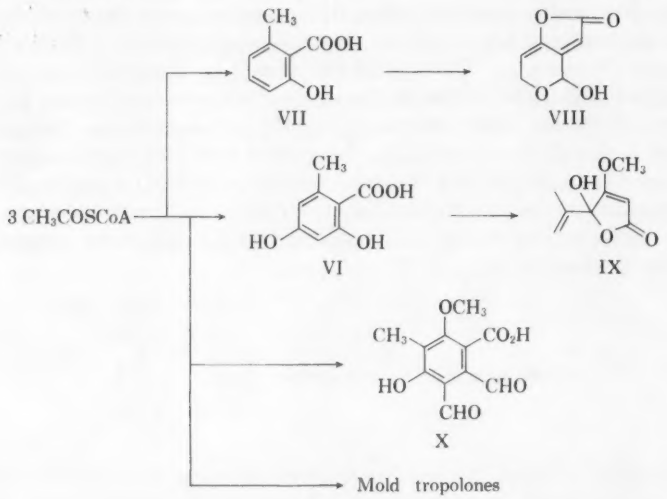
There remains, of course, the ambiguity about the origin of C_1 and C_7 mentioned earlier. Of the two alternatives, we prefer the one presented here because Bentley[20] has shown that C_9 of stipitatonic acid is derived from the carboxyl carbon of acetate. Thus, stipitatonic acid has the following distribution of acetate and formate carbons based on a reinterpretation of Bentley's earlier work:[14]



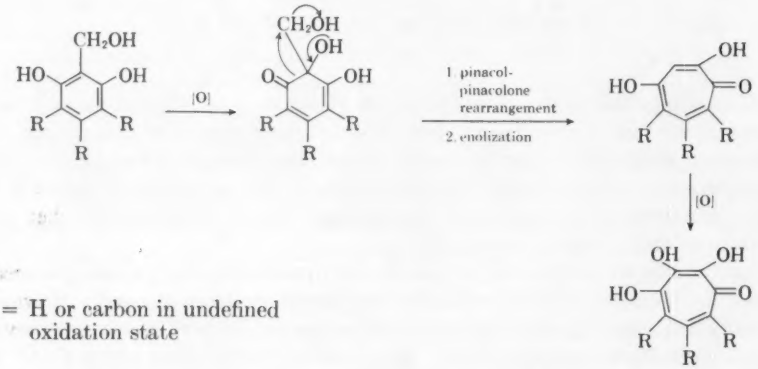
Since, in stipitatonic acid, a carboxyl carbon of acetate is attacked at C_5 we believe it more likely that the additional one carbon fragment present in puberulonic acid is attacked at C_3 rather than replacing the acetate carboxyl carbon at C_5. This raises the possibility of a ten-carbon intermediate in the biosynthesis of puberulonic acid. Tannenbaum has described[15] the isolation from *P. stipitatum* of what may prove to be such a ten-carbon tropolone.

The route by which the mold tropolones are formed from their simple precursors cannot be discussed in detail with only the present evidence at hand. However, an intriguing series of structural relationships can be deduced, which will provide a basis for further experimentation. The condensation of three acetyl CoA[21] (or malonyl CoA[22]) fragments to produce orsellinic acid (VI) is a process that finds experimental justification in the work of Birch, particularly his studies on the bio-

synthesis of 6-methylsalicylic acid (VII).[23] This latter substance has been shown by the beautiful work of Tannenbaum and Bassett[24] and of Bu'lock and Ryan[25] to undergo an oxidative ring fission and so to give rise to patulin (VIII). A similar ring fission reaction has been postulated[26] to explain the biosynthesis of penicillic acid (IX). Birch has suggested[27] that the acquisition of two one-carbon units by orsellinic acid or a derivative can lead to such ten-carbon substances as cyclopaldic acid (X).



We should like to suggest that an oxidative ring enlargement of a substance such as cyclopaldic acid is the biosynthetic pathway by which tropolones are produced. One of the many detailed mechanisms that can be envisioned to effect this change is the following:



For stipitatonic acid a scheme of the above kind operating on the appropriate nine-

carbon precursor will produce an intermediate with the correct level and site of nuclear oxidation. Another attractive mechanism for oxidative ring enlargement would involve an oxidative ring fission followed by a subsequent recyclization of the acyclic intermediate. Indeed, such a scheme is in closer formal analogy to the mechanism of ring opening that produces patulin.

It is impossible to say whether or not the extra carbon is lost in puberulonic acid biosynthesis before or after ring enlargement. Many such questions as to sequence of transformations, precise structure (including appropriate biological activation) of intermediates, etc., must await further experimental developments for their solution.

The isolation by Bentley and Thiessen[28] of an enzyme system from *P. stipitatum* that catalyzes the decarboxylation of the nine-carbon tropolones to their eight-carbon analogues makes it virtually certain that the nine-carbon stipitatonic acid and puberulonic acid are the immediate precursors of their eight-carbon analogues, stipitatic acid and puberulic acid.

An interesting possible confirmation of the suggestion that penicillic acid belongs in the same biosynthetic region with puberulonic acid and puberulic acid is the fact that all three substances have been isolated from the same strain of *P. puberulum*.[1]

Summary.—The mode of incorporation of certain labeled substrates into the mold tropolones, puberulonic acid, and puberulic acid has been studied. The origin of the various carbon atoms of the tropolones has been determined, showing that these substances are probably built up directly from simple one- and two-carbon precursors. It has been proposed that the mold tropolones are biogenetically related to benzenoid metabolites such as orsellinic acid and cyclopaldic acid.

* Presented at the 137th meeting of the American Chemical Society, Cleveland, Ohio, 1960; cf. Abstracts of papers 39–0. A preliminary communication of some of these results has appeared in *Biochem. Biophys. Res. Comm.*, **2**, 107 (1960).

† Aided by a grant from the Brown Hazen Fund of the Research Corporation.

‡ Contribution No. 2630.

§ R. Bentley and J. Murray have both independently suggested in personal communications the possibility of such a relationship.

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*ON THE ELECTRON DONATING PROPERTIES OF CARCINOGENS**

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A. and B. Pullman[1] have shown that polynuclear carcinogenic aromatic hydrocarbons could be characterized by the presence of a strong "K-region," provided that the action of this region was not compensated by the presence of an active "L-region." These authors also showed that the energies of the highest filled orbitals of these carcinogens were low. The energy of the highest filled orbital was characterized by the Pullmans by the "K-value" which is a linear function of the ionization potential. It is rather unfortunate that these authors should have chosen the same letter for the characterization of a region (two C-atoms) and an energy value, which, inevitably, has to lead to confusion. For this reason, in the present paper, we will use the letter P (in honor of the Pullmans) to characterize the energy of the highest filled orbital or the lowest empty orbital. The P for the highest filled will, according to the original denotation, have, as a rule, a plus; the P for the lowest empty orbital, a negative sign.† This theory was, for the time being, limited to polynuclear aromatic hydrocarbons. Kofahl and Lucas found a certain parallelism between carcinogenic activity and the affinity of the hydrocarbons for the Ag ion.[2] Weil-Malherbe[3] and others found, in certain cases, a tendency for complex formation with purines. Mason[4] pointed to certain relations of orbital energies. None of these theories could, till now, be extended to a wider variety of carcinogens, belonging to various chemical groups, and so it may not be superfluous to look for other connections between carcinogenicity and chemical properties.

It was suggested, in a previous paper,[5] that indoles owe their strong electron donor properties to the strong "local" donating property of certain C atoms, characterized by a high negative formal charge.[6] These C atoms, being part of a conjugated system, might then draw for the electrons, to be donated, on the π pool of

the whole molecule. This strong "local" electron donating property of the indoles was demonstrated by the formation of a black charge transfer complex with iodine, I_2, which complex gave a very high and narrow electron spin resonance (ESR) signal which indicated that a whole electron was given off by the donating indole.

Since the carcinogenic aromatic hydrocarbons, studied by the Pullmans, also have a high electron density in their K-region, which is conjugated with the rest

TABLE 1

	Black I_2 complex	Color with trinitrobenzene	Carcinogenicity	P	Number of spins/gram
Biphenyl	0	Very faint yellow	0	0.705	...
2-Aminobiphenyl	0	Light yellow	0	...	...
4-Aminobiphenyl	+	Purple	+	0.460	...
Naphthalene	0	Yellow	0	0.618	...
1-Naphthol	0	Orange-yellow	0	0.519	...
2-Naphthol	0	Yellow	0	0.569	...
1-Naphthylamine	0	Red	?	0.394	...
2-Naphthylamine	0	Red	?	0.460	...
1-OH-2-Naphthylamine	++	Red*	++	0.334	...
Indole	++	Yellow	0	0.534	1×10^{19}
Fluorene	0	Yellow	0	0.695	...
2-Aminofluorene	+++	Dark purple	++	0.450	1×10^{18}
4-Aminofluorene	0	Orange	0	...	...
Azobenzene	0	0	0	0.634	...
p-Aminoazobenzene	0	Orange	0	0.451	...
p-Dimethylazobenzene	++	Purple	++	...	4×10^{18}
O-Aminoazotoluene	++	Red-brown	++	...	1×10^{19}
Acridine	0	Yellow	0	0.610	...
Transstilbene	0	Yellow	0	0.503	...
Anthracene	0	Orange	0	0.414	...
Phenanthrene	0	Yellow	0	0.605	...
Triphenylene	0	Yellow	0	0.684	...
Terphenyl	0	0	0	0.593	
Chrysene	0	Yellow	0	0.520	...
Naphthacene	‡	Red-brown	0	0.295	...
Benzanthracene	0	Orange	?	0.452	...
10-Methylbenzanthracene	+	Red	++	0.415	...
9,10-Dimethylbenzanthracene	++	Red	+++	0.387	1×10^{19}
Pyrene	0	Yellow	0	0.445	...
1,2-Benzpyrene	0	Yellow	0	...	...
3,4-Benzpyrene	+++	Red	+++	0.372	1×10^{18}
Methylcholanthrene	+++	Dark purple	+++	0.388	3×10^{18}
1,2-5,6-Dibenzanthracene, insoluble	...	...	+	0.473	...
Picene (0.01 *M*)	0	Yellow	0	0.501	...
Pentacene, insoluble	...	...	...	0.220	...
Perylene	‡	Brown-red	0	0.347	...
Coronene (0.025 *M*)	0	Orange	0	0.539	...
Phenothiazine	+++	Blue	0	−0.210	*
Methyleneblue†	+++	?	0	0.398	*
Acridineorange†	+++	?	0	0.657	*
1,10-Phenathroline	+++	0	...	0.649	...

* Color doubtful because of strong own color.
* Substances give ESR signal without I_2.
† Precipitation with Lugole solution in aqueous medium.
‡ Insoluble in 0.1 *M*, crystals turn black with I_2.

Column 2: Whatman 1 filter paper was moistened with an 0.1 *M* solution of substance noted in Column 1. As solvent, mostly chloroform was used. In case of less soluble substances, xylene, and exceptionally (1-OH-naphthylamin) methanol was used. After evaporation of the solvent, an 0.1 *M* I_2 solution in chloroform was allowed to run out of a pipette, in the form of a strip, onto the paper (Fig. 2). One cross means the development of a short-lived black color after evaporation of the chloroform. Three crosses mean that black color developed even before evaporation of the chloroform. Brown, or other colors, were disregarded. In Column 2 are given the colors obtained when an 0.1 *M* sym. trinitrobenzene solution (in chloroform) was allowed to evaporate after having dropped on the filter paper treated with the various substances as above. In Column 3 the carcinogenicity is noted, as reflected by the literature. Column 4 gives the P-value of the highest filled molecular orbital, as taken mainly from the works of the Pullmans. In the last column is given the ESR signal. All black I_2-complexes of carcinogens gave a very high and narrow signal, the *g* value of which corresponded approximately to that of a free electron while the I_2 complex of phenanthroline gave no signal at all. The numbers give the number of spins per gram of the complex. The measurements were made at room temperature. The ESR signal being temperature dependent, the numbers quoted give only the order of magnitude. The pure carcinogens or I_2 in itself gave no, or only a very faint, signal.

of the molecule, it follows that if our theory of indoles was correct, these hydrocarbons should also form a black charge transfer complex with iodine, which complex should also give a high ESR signal. In case that the strong electron-donating property revealed by this reaction is connected with carcinogenicity, we should expect that carcinogens belonging to other chemical groups like azobenzenes, fluorenes or biphenyls, should form a similar black charge transfer complex with iodine, while their noncarcinogen homologues should not give such a reaction. For this reason we tested a number of substances for their reaction with iodine to find out whether there is an interrelation between carcinogenicity and the formation of a black complex with iodine, that is, the ability to donate an electron to a suitable local acceptor. The results of these experiments are given in Table 1.

The results of these experiments can be summed up by saying that all the strong carcinogens tested gave a black complex, while their noncarcinogenic homologues gave no such reaction. The experience seemed to hold for different substance groups. This makes it seem possible that *carcinogenicity of these substances is connected with their ability to form strong charge transfer complexes with local acceptors and give off an electron.* Needless to say, carcinogenicity involves various parameters and so a black I_2 charge transfer complex does not necessarily indicate carcinogenicity, as shown by the formation of such complexes by the noncarcinogenic indoles. All the same, the close relation of charge transfer with carcinogenicity is strongly suggested by our results and can most clearly be demonstrated by comparing homologues, possibly isomers, thus closely related substances, some of which are carcinogenic, and some of which are not, or only very weakly so.

The filter paper shown in Figure 1 was moistened under "a" with an 0.1 *M*

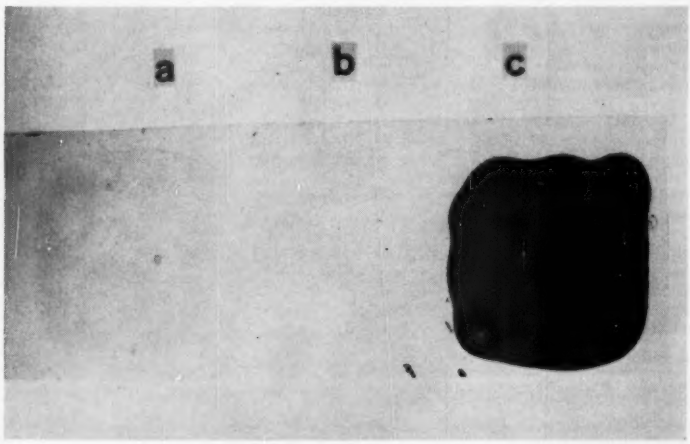

FIG. 1.

chloroform solution of pyrene, under "b" with a similar solution of 1,2-benzpyrene, and under "c" with 3,4-benzpyrene. After the chloroform evaporated, the paper was exposed, for a few minutes, to the action of I_2 vapour.[7] As the figure shows, the first two substances which are not carcinogenic, and have no strong K-region, gave no reaction, while the third, a strong carcinogen, turned black.

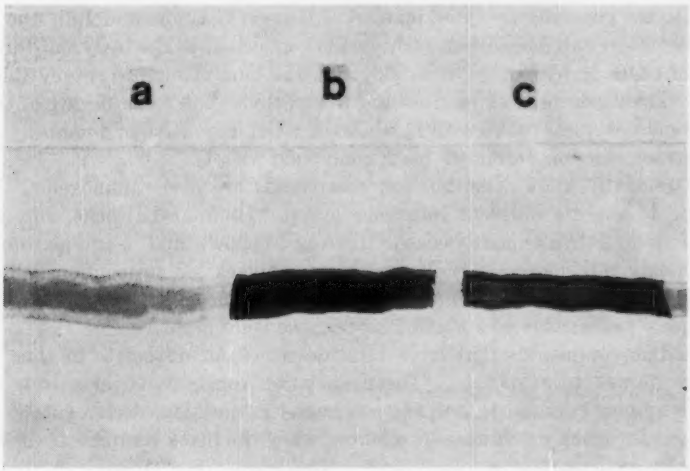

FIG. 2.

A similar experiment is shown in Figure 2, where the paper was moistened under "a" with an 0.1 *M* chloroform solution of benz(a)anthracene, which is not carcinogenic or only very poorly so. Under "b" a similar solution of the strongly carcinogenic 9,10-dimethylbenzanthracene (7, 12-dimethylbenz(a)anthracene) was employed. For comparison, the paper was moistened under "c" with an 0.1 *M* solution of 3 methylcholanthrene. After the chloroform evaporated, a line was drawn with a pipette containing 0.1 *M* I_2, dissolved in chloroform. As the figure shows, no black compound was formed by the noncarcinogenic benzanthracene, while the two other substances, both strong carcinogens, formed a black compound. I owe the recrystallized preparation of 7,12-dimethylbenz(a)anthracene (MP 122–23) to the generosity of Dr. Charles B. Huggins, while the methylcholanthrene was kindly supplied by Hoffman-LaRoche, Inc.

The monomethylbenzanthracenes are much less carcinogenic than the 9,10-dimethyl derivative. Owing to the generosity of Dr. Melvin S. Newman, we are in possession of the whole series of monomethyl-1,2-benzanthracenes.‡ Only one of them is known to be strongly carcinogenic, the 10-methyl-1,2-benzanthracene. This gives a black reaction with iodine, if treated in a fashion similar to the 9,10-dimethyl derivative. The others give no, or only doubtful, reactions.

The noncarcinogenic fluorene does not give a black I_2 compound. Owing to the generosity of John H. Weisburger, 2 and 4 aminofluorene could be tested, the first of which is strongly carcinogenic, while the latter is not. The carcinogenic 2-amino derivative gave a very strong reaction, while the 4-amino derivative was negative showing only a brown coloration with I_2, as given by many different substances. Dr. Weisburger provided also 2- and 4-aminobiphenyl, the first of which is not carcinogenic, the latter moderately so. The first gave only an uncharacteristic brown color, while the latter became greenish black on treatment with I_2.

An interesting instance is that of naphthylamines. As is generally known, β-naphthylamine causes cancer of the bladder while the α form is inactive. Ac-

cording to the generally accepted opinion of Bonser, Clayson, and Jull,[8] the naphthylamine is not carcinogenic as such, but is converted, in the body, into the carcinogenic 1-OH derivative. Dr. E. Boyland was kind enough to supply this substance. The experiment showed α- and β-naphthylamine to be negative, that is, to give only untypical colors with I_2, while the 1-OH derivative of β-naphthylamine gave a strong reaction, forming a black compound with I_2.

As is generally known, certain azo compounds are also carcinogenic, though belonging to a quite different substance group. Hoffman-LaRoche, Inc. kindly supplied *p*-dimethylaminoazobenzene (Butter Yellow) and *o*-aminoazotoluene, both strongly carcinogenic. Both gave a strong black iodine reaction, indicating that this reaction is actually connected with the carcinogenic property. The noncarcinogenic azobenzene and *p*-aminoazobenzene were negative.

The iodine compounds that have been described all dissociate in time if the iodine is allowed to evaporate. The dissociation constants cover a wide range. A strong affinity between I_2, and the compound in question, declares itself in the formation of a black precipitate in solution, while the black complex maintains its black color on paper for a longer time. The black color appears on the paper before the solvent evaporates. In this case the black complex will also be formed rapidly if the compound, dried on paper, is exposed to iodine vapour. The iodine complex, in this case, can be prepared by simply mixing an 0.1 *M* solution of the substance with the double volume of 0.1 *M* I_2, both dissolved in chloroform. If the substance has less affinity to iodine, then the black complex may be obtained by cooling the mixture, while, on warming, the complex dissolves again showing the reversibility of the reaction. In this case, the black color will appear on paper, moistened with a chloroform solution of the substance (0.1 *M*) and I_2 (0.1 *M*) only after the solvent has evaporated. If the affinity is rather low, the black color will very soon disappear, owing to its dissociation and evaporation of the I_2. In this case, in order to make the complex preparatively, rather low temperatures have to be applied, and no black complex can be obtained by exposing the paper, treated with the carcinogen, to the vapours of I_2.§

Discussion.—The foregoing strongly suggests that carcinogenicity, in the substances studied, is connected, in some way, with their ability to form a strong charge transfer complex with I_2 which gives a strong ESR signal. But the black reaction of the noncarcinogenic substances, like indole, phenanthroline, thiazine, shows that other molecular parameters must also be involved. Why the higher aromatics, as perylene, violanthrene, which give a black I_2-complex, are not carcinogenic, might be explained by the absence of a K-region, their very poor solubility, or the presence of strongly developed L-regions. Another factor, possibly involved in carcinogenicity, as pointed out by the Pullmans, may be the low P-value. Carcinogenic hydrocarbons are also characterized by a low P-value. Indoles, which form a black charge transfer complex with I_2, but are not carcinogenic, do not have a low P-value. The same is true for phenanthroline. The ionization potential, or P-value, declares itself also in the color of the charge transfer complex with trinitrobenzene. In case of a high P, the high energy of blue light is needed to transfer the electron, and thus the charge transfer complex is yellow. As we go, gradually, to substances of lower P, the color changes from red-orange to red or purple, which color indicates that relatively little energy is needed to transfer the

electron. Fujimori's curve,[9] with its curvature, suggests that in this region the transfer may be spontaneous and may need no external energy at all. As can be seen in Table 1, the strong carcinogens studied all show red or purple charge transfer colors, and have thus a low P-value, while the noncarcinogen indoles, or aromatics like chrysene or anthracene, give a yellow color. It seems reasonable to suppose that the "local" electron donation has to be backed up by a low P-value of the highest filled molecular orbital in order to lead to carcinogenicity. The relatively high P-value of indoles, and the corresponding yellow color given with trinitrobenzene, indicates that the π pools of these molecules part less readily with their electrons, thus do not have the intense donor property demanded for carcinogenicity, which demands both a low P as well as a high local electric charge which enables the molecule to enter into a local electron transfer.

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† Only in exceptional cases does the highest filled bonding orbital acquire a minus (−) and the lowest empty antibonding orbital a plus (+) sign.

‡ 2′ Methyl, 3, Methyl′ 4′ Methyl, 3′ Methyl, 4-Methyl, 5-Methyl, 6-Methyl, 7-Methyl, 9-Methyl, and 10-Methyl 1,2-benzanthracenes.

§ Difficulty was experienced in preparing the I_2-complex of Butter Yellow and aminoazotoluene because at the low temperatures required the I_2 crystallized out. The black complex was prepared by dissolving the carcinogens in a small volume of benzene, adding 2 moles of I_2 per carcinogen molecule in the form of a 0.1 *M* benzene solution, and storing the mixture at −20°C overnight. The benzene solidified, enclosing the black I_2 complex. Then an equal volume of pre-cooled hexane was added, and the systems allowed to warm to 0°C in the refrigerator. The benzene dissolved, and the I_2 complex was centrifuged out at 0°C, exposed to short evacuation and air drying. Dimethylbenzanthracene, which has even less affinity to I_2, separated in crystals on cooling. In this case, a 0.1 *M* solution of the hydrocarbon was prepared and a double volume of a 0.1 *M* of I_2 was added, chloroform being used as solvent. The chloroform was sucked off by a water pump warming the solution in tepid water. A granular black mass was left behind which quickly lost I_2 on warming or storage.

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AMINO ACID INCORPORATION INTO PROTEINS BY ESCHERICHIA COLI RIBOSOMES

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Evidence that ribosomes are an important site of protein synthesis has come mainly from experiments with animal cells. It has been shown *in vivo* that C^{14} amino acids are incorporated into peptide linkage first in ribosomes, and by addition of cold amino acids, they can be chased to the bulk of the proteins of the cell.[1–3] *In vitro*, they become bound in ribosomal proteins[2] or, after their passage on ribosomes, in newly synthesized proteins.[4]

Similar evidence has recently been obtained with *Escherichia coli*. It has been shown with intact cells that sulfur is incorporated first into ribosomal protein, and can be chased from there to other proteins of the cell.[5] Lamborg and Zamecnik[6] have found that a cell-free system, containing ribosomes and soluble factors, incorporated amino acids into particles and soluble proteins at a rate several fold higher than do rat liver preparations. Like the liver system,[7, 8] the system from *E. coli* required ATP and GTP. That the incorporation was into peptide linkage was shown in partial hydrolysates, in which the labeled leucine appeared in four peptide fractions separable by paper chromatography.

There are four kinds of ribosomes in cell free extracts of *E. coli*, characterized by sedimentation coefficients of 30 S, 50 S, 70 S, and 100 S.[10] At low magnesium concentration, only the 30 S and 50 S ribosomes are present; on raising the level of magnesium, one 30 S and one 50 S combine in a 70 S particle; at still higher magnesium concentration, two 70 S form a 100 S dimer.

In the work described here, a system similar to that of Lamborg and Zamecnik[6] has been used, and the process of incorporation into 30 S, 50 S, 70 S, and 100 S ribosomes has been analyzed by the sucrose gradient technique.[5]

Materials and Methods.—*E. coli* strain B was grown at 37°C, with aeration, in broth. The bacteria were harvested when they had reached about 5×10^7 cells per ml, that is about 2 gm wet weight per 10 l of culture medium. They were washed twice with the buffer used to make the extract, ground by hand immediately in a mortar for 2–3 min with 2.5 parts (wt/wt) of alumina powder[9] (Norton levigated alumina from Norton Abrasives, Worcester 6, Mass.) and extracted with 3 vol of 0.005 *M* tris buffer, pH 7.3, containing magnesium acetate at the concentration indicated in the results section. All manipulations were carried out at 4°C. The mixture of alumina, broken cells, and buffer was centrifuged at 8,000 *g* for 15 min. The sediment was composed of two layers: a lower layer of alumina and some intact cells, and a brown upper layer of large cell debris. The supernatant was once more centrifuged at 8,000 *g* for 15 min to sediment remaining alumina and cell debris. It is referred to as *crude extract*. This contained the bulk of the ribonucleic acid (RNA) of the cell, in the form of ribosomes and soluble RNA; soluble enzymes and other soluble factors;[10] and a small amount (less than 5% of the total dry weight of the extract) of cell debris, deriving probably from cell membranes and cell walls.[11]

To isolate 30 S and 50 S ribosomes quickly so that the preparations could be tested within 24 hr of the time when the bacteria were harvested, the following procedure was used.

The crude bacterial extract was prepared in 0.005 *M* tris buffer, pH 7.3, containing 0.005 *M* Mg^{++}. It was then centrifuged for 90 min at 125,000 *g* in three 5 ml centrifuge tubes in the swinging bucket rotor SW 39. Four ml supernatant fluid were carefully removed from the top of each tube, recentrifuged for 210 min at 105,000 *g*, and the top $^2/_3$ were pipetted off to form the *supernatant* fraction. Each of the 5 ml centrifuge tubes, with the pellet, was drained of remaining supernatant and rapidly rinsed with about 1 ml 0.005 *M* tris buffer with a small glass rod. The pellets were then resuspended in this buffer with a small glass rod. The preparation was made up to 23 ml with the buffer, centrifuged first 15 min at 8,000 *g* to remove aggregated particles, then at 105,000 *g* for 300 min in two 12-ml tubes in the "40" angle rotor. About $^9/_{10}$ of the supernatant was carefully removed and discarded, while the remaining liquid was swirled around on the top of the pellet for 2 to 4 sec to bring the 30 S particles into solution. This was then poured off to give the 30 S fraction.

The remaining pellet was resuspended in 23 ml 0.005 *M* tris buffer containing 0.001 *M* Mg^{++}, and centrifuged first 15 min at 8,000 *g* to remove aggregates, then 120 min at 105,000 *g* in the "40" rotor. The supernatant was poured off and the pellet was resuspended and recentrifuged once more at low (8,000 *g*) and high (105,000 *g*) speed as above. The pellet was finally resuspended in 2–3 ml tris buffer with 0.001 *M* Mg^{++}, and centrifuged 15 min at 6,000 *g*. The supernatant formed the 50 S preparation.

Both the 30 S and 50 S solutions were examined immediately in the analytical centrifuge with ultraviolet optics to determine the amount of contaminating component. The preparations were then tested for the incorporation of amino acids.

After the reaction for the incorporation of labeled amino acids, the samples were washed with cold and hot trichloroacetic acid (5%), ethanol, and ether, and plated as described by Siekevitz.[12] They were counted in a gas flow counter with an end window. In all figures, counts are given as counts per min; elsewhere they were corrected for the efficiency of the counter and are therefore expressed as disintegrations per min (d.p.m.). The samples obtained from sucrose gradient experiments, or from the elution of a DEAE-cellulose chromatogram, were precipitated and washed with cold 5% trichloroacetic acid on Millipore methyl-cellulose filters.

A mixing device, of the type commonly used for gradient elution from chromatographic columns, delivered 24 ml of a sucrose gradient ranging from 20% at the bottom to 5% at the top of a 25 ml nitrocellulose centrifuge tube. One ml of the dialyzed reaction mixture (see *Results*, section *h*) was layered on top of the gradient. It was then centrifuged in the SW25 rotor of the Spinco centrifuge at 25,000 r.p.m. for 150 min. A pinhole was made at the bottom of the centrifuge tube and drops were collected for measuring both the radioactivity and the optical density at 260 mμ.

A model L Spinco ultracentrifuge was used to fractionate the *crude extracts*. The centrifugal forces given represent *g* average, as calculated for the middle of the centrifuge tube. Analytical ultracentrifugation was done with a model E Spinco centrifuge, with ultraviolet optics. The films taken were analyzed for

their optical density with a Spinco Analytrol densitometer. The sedimentation constants are expressed in Svedberg units.

Proteins were estimated by the Folin phenol reagent.[13]

Adenosine triphosphate (ATP), guanosine triphosphate (GTP), and tris (hydroxymethyl) aminomethane were obtained from Sigma Chemical Company, St. Louis, Mo., phosphoenol pyruvate and pyruvate kinase from C. F. Boehringer und Sohne, Mannheim, Germany, pancreatic ribonuclease (RNase) and deoxyribonuclease (DNase) from Worthington Biochemical Corporation, Freehold, N. J. Individual L-amino acids (all C^{14} labeled with the exception of S^{35} cysteine) were obtained from Schwartz Laboratories, Mt. Vernon, N. Y., and C^{14} chlorella protein hydrolysate (250 μc/mg) from Volk Radio-Chemical Company, Chicago, Ill. Millipore filters, from Millipore Filter Corporation, Bedford, Mass., were used in sucrose gradient experiments. Diethyl aminoethyl cellulose (DEAE) was obtained from Brown Co., Berlin, N. H.

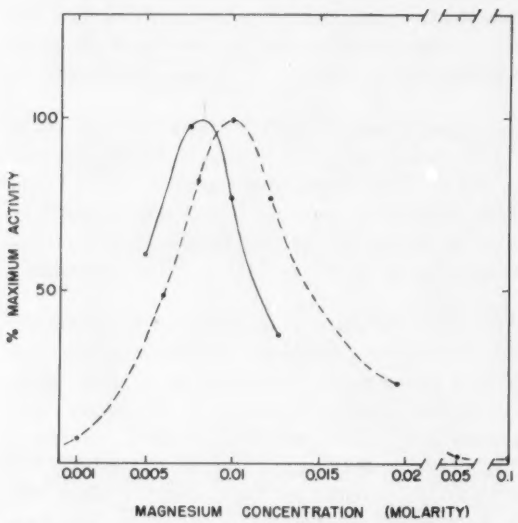

FIG. 1.—Per cent of maximum activity as a function of Mg^{++} concentration, with two different *crude extracts*.

Results.—(*a*) *Conditions for incorporation of amino acids:* The reaction mixture contained 1 μmole ATP, 5 μmoles phosphoenol pyruvate, 40 μg pyruvate kinase, 0.022 *M* KCl, 0.008 *M* magnesium acetate, 1 μmole C^{14} alanine or leucine (3×10^5 d.p.m.) and 0.5 ml *crude* bacterial *extract* (about 6 mg. soluble proteins and 3 mg ribosomes) in 0.85 *M* tris buffer, pH 7.3. The results shown in Table 1 confirm Lamborg and Zamecnik's[6] conclusion that the *E. coli* system has about the same requirements as that from rat liver.[7, 8] With *crude extract*, however, the addition of GTP and a supplementary amino acid mixture did not increase the incorporation of amino acids (see section *f*).

The level of incorporation of amino acids achieved was very sensitive to the concentration of magnesium (Fig. 1). With 0.001 *M* Mg^{++}, the activity of the system reached only about 7 per cent of its maximal value which was obtained at a Mg^{++} concentration varying between 0.007 *M* and 0.011 *M* with different preparations. The activity of the system did not seem to depend upon the Mg^{++} concentration of the buffer used to make the cell-free extract, but rather on the amount of Mg^{++} present in the reaction mixture: the same level of activity was achieved when extracts made in 0.001 *M*, 0.005 *M*, and 0.01 *M* Mg^{++} were tested in 0.01 M^{++}.

When 0.01 *M* calcium was used to replace Mg^{++} in preparing the extract and

TABLE I

INCORPORATION OF C^{14}-ALANINE INTO *Crude Extract*, *Supernatant*, AND *Ribosome* FRACTIONS

	d.p.m.	d.p.m./mg dry weight
Crude extract (0.5 ml, 3 mg ribosomes)	8300	1080
Same without ATP, PEP, and PK	100	13
Same without PEP and PK	139	18
Same with 1×10^{-4} *M* Mg^{++}	116	15
Supernatant (0.1 ml)	41	51
Ribosomes (3.2 mg)	423	132
Ribosomes (3.2 mg) + *supernatant* (0.1 ml)	2900	725

The complete reaction mixture consisted of 1 μmole ATP, 5 μmole phosphoenol pyruvate (PEP), 40 μg pyruvate kinase (PK), 0.022 *M* KCl 0.01 *M* magnesium acetate, 1 μmole C^{14}-alanine (3×10^5 d.p.m.) and the cell fraction in a total volume of 0.85 ml. Incubation at 37°C for 45 min. Radioactivity measured in precipitate from hot trichloroacetic acid.

testing it, only about 10 per cent of the activity obtained with 0.01 *M* Mg^{++} was observed. Calcium (0.01 *M*) was able to increase the activity of an extract containing 0.001 *M* Mg^{++} to about half the level achieved with 0.01 *M* Mg^{++}. Manganese had an effect similar to that of calcium. It should be noted that the ATP generating system requires Mg^{++} at an optimum concentration of 0.004 *M*.[14]

(*b*) *Stability with time:* The activity of the *crude extract* in the incorporation of amino acids was not modified after 6 hr at 4°C. At 20° or 25°C, it remained constant for 2 hr. When kept at 4°C for 24 hr, a decrease in activity, varying with different preparations from less than 10 to about 30%, was observed. The same pattern of stability was obtained with isolated *ribosome* preparations.

When *crude extract* was kept frozen at −15°C, the results were somewhat variable: in some cases there was no loss of activity after 72 hr; in other instances a preparation kept frozen for 6 hr had lost 40 per cent of its activity. The mode of freezing and thawing might have been responsible for these variations.

(*c*) *Fractionation of the system:* The *crude extract* was centrifuged for 90 min at 125,000 *g*. The upper half of the supernatant was carefully pipetted off and recentrifuged for 180 min at 100,000 *g*. The upper $^2/_3$ of the supernatant were pipetted off to yield the fraction referred to as *supernatant*.

The pellet obtained by centrifuging the *crude extract* was drained of all supernatant, and the centrifuge tube with the pellet was rapidly rinsed with 1 ml. tris magnesium buffer. The pellet was resuspended with a glass rod in tris magnesium buffer to the original volume of the extract. The suspension was centrifuged at 8,000 *g* for 15 min to remove aggregated particles and then at 125,000 *g* for 90 min. The pellet was again resuspended in tris magnesium buffer, and the suspension centrifuged at 8,000 *g* for 15 min. The supernatant was poured off to yield the *ribosome* fraction.

The *cell debris* fraction consisted of pieces of cell walls and cell membranes which sedimented as a brown layer on top of the alumina during the first centrifugation after grinding the cells. To obtain this fraction free of crude extract and intact cells, it was washed four times in the centrifuge with the buffer used to make the extract. Each time only the upper layer of the pellet was resuspended, in order to eliminate the intact cells which sediment faster to the bottom of the tube.

The results presented in Table 1 show that the system requires *ribosomes* and a small amount of soluble factors present in the *supernatant*.

It is seen in Table 2 that: (*a*) the *cell debris*, formed mainly of cell walls and cell membranes, did not incorporate amino acids at a significant rate, either in the

presence of Mg^{++} alone, or with both Mg^{++} and manganese; (*b*) the addition of *supernatant* did not produce any marked change, and the incorporation obtained with *crude extract* was not increased by addition of *cell debris;* (*c*) when the *crude extract* was centrifuged for 45 min at 25,000 *g* which sedimented most of the small amount of *cell debris* present, the specific activity did not decrease, but rather increased slightly, indicating that cell debris are not involved in the incorporation of amino acids in the *crude extract.*

TABLE 2

INCORPORATION OF C^{14}-ALANINE IN PRESENCE OF MG^{++} AND MN^{++} IONS*

	Mg^{++} (molarity)	Mn^{++} (molarity)	d.p.m.	d.p.m./mg (dry weight)
Cell debris†	0.001	....	34	14
Cell debris	0.008	....	50	21
Cell debris	0.001	0.001	40	17
Cell debris	0.001	0.008	76	32
Supernatant‡	0.001	....	88	31
Supernatant	0.001	0.001	81	29
Cell debris + supernatant	0.001	....	120	22
Cell debris + supernatant	0.001	0.001	88	16
Crude extract	0.001	....	720	107
Crude extract	0.008	....	6840	1140
Crude extract (centrifuged 45 min at 25,000 *g*)	0.008	....	6020	1250
Crude extract + cell debris	0.008	....	6730	800

* Reaction mixture as in Table 1, except for divalent cations.
† A fraction which is composed mostly of cell membrane and cell wall.
‡ Consisting of soluble factors.

These results clearly show that under the conditions used here, the presence of a small amount of *cell debris* (<5%) in the *crude extract*[11] had no effect on the amino acid incorporation observed with the latter. This incorporation system therefore differs from that of Nisman[16] and Spiegelman,[17] who worked with *E. coli* preparations, and from that of Hunter *et al.*,[18] who used lyzed protoplasts from *Bacillus megaterium.* Under the experimental conditions reported here, *ribosomes* were essential, and the *cell debris* fraction, with or without manganese ions, had no effect.

(*d*) *Time course of incorporation of amino acids:* The reaction at 37°C was stopped at 30 sec, 2 min, 8 min, 20 min, and 45 min by addition of 2.5 μg ribonuclease per ml (see section *g*), and the particles were separated from the soluble phase by high speed centrifugation. The results are shown in Figure 2. The rate of the reaction dropped quickly: after about 4 min half the maximum level of incorporation had already been achieved and the curve leveled off at about 45 min. As seen in a number of experiments, the curves of the incorporation into ribosomes and soluble proteins had about the same shape. The amount of radioactivity in the soluble fraction was 25 to 35 per cent that found in the ribosomes.

When, at any time during incubation, *supernatant* was added to a reaction mixture containing *crude extract,* it had no effect on the incorporation. In contrast, added washed *ribosomes* produced a rise which followed essentially the same course as did the *crude extract* in the experiment shown in Figure 2. Thus the incorporation of amino acids does not stop because any of the soluble factors present in the beginning of the reaction is missing after 45 min, but rather because the particles themselves are no longer able to function.

(*e*) *Time sequence of incorporation in cell fractions:* The incorporation reaction was carried out with 0.5 ml crude extract and 2.5 μc C^{14} chlorella hydrolysate (12.5 μg) at 37°C, in duplicate. After 10 sec, 0.2 μmole of each of the 18 amino acids (a total of about 200 μg) was added to sample 1, while sample 2 received 5 μg ribonuclease to stop the reaction. After 45 min the samples were cooled and centrifuged at high speed to separate ribosomes from soluble proteins. The two fractions were then treated by the Siekevitz procedure[12] and counted. The soluble proteins from sample 1 had 7,100 d.p.m., while the ribosome fraction had only 900 d.p.m. In sample 2, there were 1,200 d.p.m. in the soluble phase and 1,810 in the ribosomes. Thus C^{14} amino acids had been chased from the particles to the soluble proteins. The reason why sample 1 had a total of 8,000 d.p.m. while sample 2 had only 3,010 d.p.m. is probably that some labeled amino acids, presumably bound

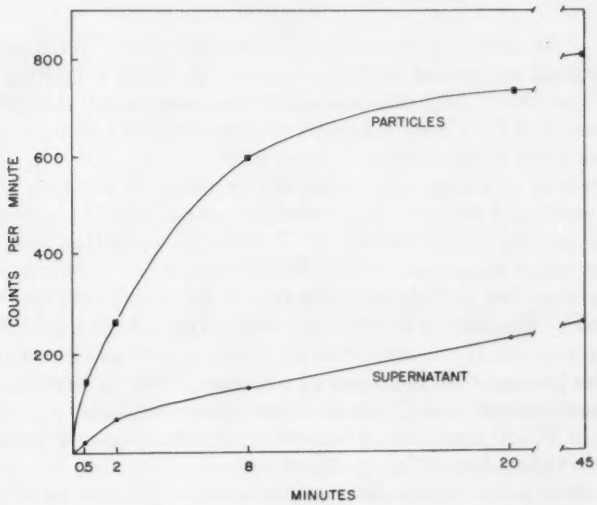

FIG. 2.—Time course of C^{14}-alanine incorporation into ribonucleoprotein particles and supernatant.

to soluble RNA and enzymes, were not diluted by addition of cold amino acids in sample 1, and therefore continued to be incorporated; in sample 2, however, it is likely that ribonuclease hydrolyzed soluble RNA and stopped at once the incorporation into particles.

After a similar experiment, the mixture was analyzed by the sucrose gradient technique (see section *h*). The results showed that the counts could be chased from *active 70 S* ribosomes to the soluble phase.

(*f*) *Amino acids incorporation:* (1) *Incorporation of individual amino acids.* Each of 18 amino acids, when added alone to the reaction mixture, was incorporated by *crude extract* as shown in Table 3. The amount incorporated was from 0.7 mμmole for tryptophan to 5 mμmoles for lysine per mg. of ribosomes present during the reaction. The high value found for lysine might be due partly to linkage by the ϵ-amino group.[15]

TABLE 3

INCORPORATION OF INDIVIDUAL C^{14} AMINO ACIDS

	Moles % amino acids Expt. 1	Expt. 2		Moles % amino acids Expt. 1	Expt. 2
Alanine	3.4	5.8	Lysine	19.5	15.4
Arginine	4.5	4.8	Methionine	2.0	2.2
Aspartic	4.2	5.7	Phenylalanine	2.9	2.9
Cysteine	2.2	2.2	Proline	4.2	4.9
Glycine	6.0	7.5	Serine	4.0	4.0
Glutamic	6.9	6.9	Threonine	11.2	10.9
Histidine	3.1	3.7	Tryptophane	2.5	2.6
Isoleucine	7.4	6.6	Tyrosine	4.0	3.8
Leucine	7.4	6.2	Valine	3.8	4.0

1 μmole of each amino acid was tested separately with two different *crude extract* preparations (experiments 1 and 2). The experimental conditions were as given in Table 1. A total of 76.4 and 111.3 μmoles of amino acids were incorporated in experiments 1 and 2 respectively. The amino acids were C^{14} labeled except for S^{35} cysteine. In this latter case, the incorporation mixture was heated for 10 min at 90°C in 2 ml mercaptoethanol before the addition of trichloroacetic acid, to remove cysteine bound by disulfide rather than peptide linkage.

(2) *Effect of the addition of a complementary mixture of cold amino acids.* With a system composed of isolated washed *ribosomes*, to which a limiting amount of *supernatant* was added (3 mg. *ribosomes* and 0.02 ml *supernatant*), a 3-fold increase in the incorporation of C^{14} alanine was obtained on addition of a mixture of 0.2 μmoles of each of the other 17 amino acids listed in Table 3.

In the presence of a larger proportion of *supernatant* to particles, or with *crude extract*, the addition of the same complementary mixture of cold amino acids did not enhance the activity of the system. It is therefore likely that the *crude extract* and the *supernatant* contain each of the amino acids in adequate amounts. Furthermore, it was observed that the incorporation of one amino acid into *crude extract* was inhibited by the addition of either one other amino acid or a mixture of several others. For example, the incorporation of C^{14} leucine (100 μg added to the reaction mixture) was lowered by 25 per cent on addition of 100 μg alanine. In another instance, the incorporation of C^{14}-alanine (100 μg) was inhibited by 27, 67, and 89% on addition of 30, 340, and 1,700 μg respectively of a complementary mixture (equal amounts by weight) of the 17 other amino acids.

(3) *Amount of protein synthesized.* An estimate of the amount of protein synthesized in the system can be obtained by summing up the values for each amino acid incorporated in the two experiments shown in Table 3. Thus 10.0 μg and 14.7 μg were incorporated in experiments 1 and 2 respectively, with about 3 mg. ribosomes. In another instance, using a C^{14} amino acid mixture of chlorella hydrolysate, it was found that 7.4 μg of amino acids had been incorporated with the same amount of ribosomes. Therefore, an average of 3.5 μg of amino acids were incorporated per mg. of ribosomes.

(4) *Chromatography of C^{14} labeled soluble proteins.* The reaction for the incorporation of amino acids was run with 10 to 15 ml *crude extract*, 5 μc of C^{14} chlorella hydrolysate (25 μg) per ml of reaction mixture, 0.001 *M* Mg^{++} and ATP, phosphoenol pyruvate and pyruvate kinase in the proportions given in section a). After 45 min incubation at 37°C, the soluble proteins were isolated by high speed centrifugation and dialyzed against 0.005 *M* tris buffer, pH 7.3. They were chromatographed on diethyl aminoethyl cellulose (DEAE),[19] eluted by an exponential sodium chloride gradient, and the samples were analyzed for protein content and radioactivity. The elution pattern (Fig. 3) shows that the curve obtained for radioactivity follows

roughly that of the protein content. These results indicate that the soluble proteins which are labeled *in vitro* represent essentially the spectrum of the soluble proteins of the cell.

(*g*) *Inhibitors:* (1) *Pancreatic ribonuclease* (1–4 μg/ml) inhibited completely the incorporation of amino acids. This inhibition took place very fast. Whether the incorporation of amino acids was stopped by ribonuclease or by trichloroacetic

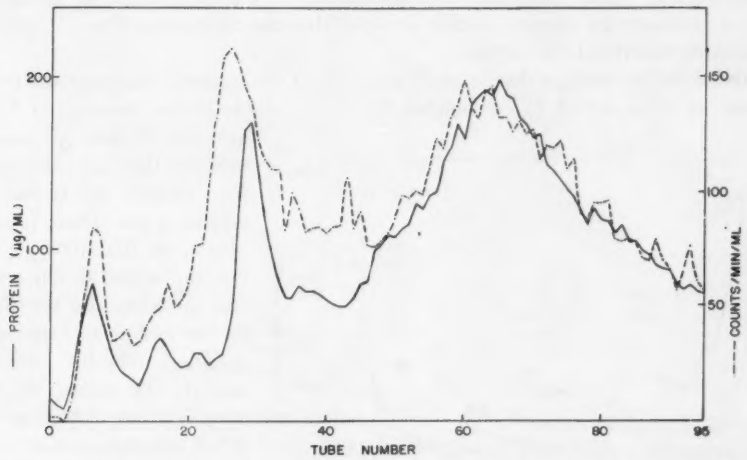

FIG. 3.—Elution pattern of *E. coli* soluble proteins by NaCl gradient from DEAE column (0.005 *M* tris, pH 7.3), 2.5 ml per collected fraction. The column had a diameter of 1 cm and a hieght of 20 cm.

acid at any time during the reaction, the same level of incorporation was obtained. Ribonuclease was therefore used to stop the reaction whenever trichloroacetic acid was unsuitable.

It is likely that this inhibition was brought about by digestion of soluble RNA, as ribosomal RNA would not be affected as rapidly under these conditions.[10]

(2) *Deoxyribonuclease* (DNase) inhibited the reaction as shown in Table 4. Several polyelectrolytes, which were found to restore the incorporation of amino

TABLE 4

INHIBITION OF THE INCORPORATION OF C^{14}-ALANINE BY DEOXYRIBONUCLEASE AND CHLOROMYCETIN

		Concentration (μg inhibitor per ml reaction mixture)	% Inhibition
Deoxyribonuclease:	added at zero time of reaction	0.1	9
		1.0	48
		5.0	70
	incubated 5 min at 28°C with *crude extract* before reaction	0.5	66
		2.5	72
	incubated 6 hr at 4°C with *crude extract* before reaction	1.0	66
Chloromycetin:	added at zero time of reaction	30.0	84
		100.0	87
		200.0	91

Reaction mixture, with 0.5 ml *crude extract*, as in Table 1. Incubation at 37°C for 45 min.

acids by isolated nuclei after DNase treatment,[20] had no effect; the DNase inhibition was not reversed by the presence in the reaction mixture of 0.5 mg of heparin, polyethylene sulfonate (molecular weight 12,600), or polyadenylic acid (molecular weight 35,000).

It seemed of interest to know if deoxyribonuclease selectively inhibits the incorporation into either ribosomes or supernatant. Thus after the incorporation in presence of 5 μg DNase, ribosomes and supernatant were separated and the measurement of radioactivity in each fraction revealed that the reaction had been inhibited to the same extent in both fractions.

This inhibition was not due to small amounts of ribonuclease contaminating the DNase, as 60 μg/ml of *E. coli* soluble RNA was stable in the presence of 5–10 μg/ml of DNase. It is also unlikely that this inhibition was caused by traces of trypsin in the DNase preparation, as 0.01–0.03 μg/ml trypsin, added to the reaction mixture, had no effect on the amino acid incorporation. Whether DNase inhibits the system by destroying the integrity of DNA remains unclear.

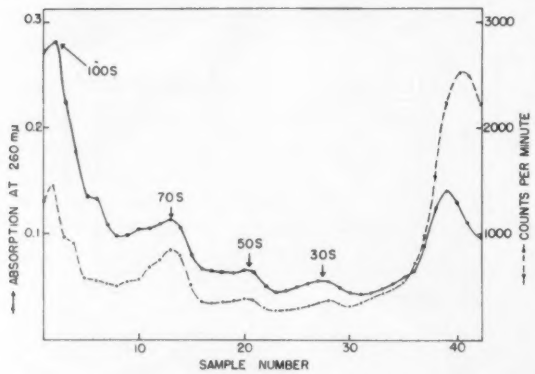

FIG. 4.—Incorporation of amino acids into *crude E. coli extract* in 0.01 *M* Mg^{++}. The reaction mixture, with 1 ml *crude extract*, 5 μc C^{14} chlorella hydrolysate, in a total volume of 1.7 ml, was incubated at 37°C for 45 min. 1 μg each of ribonuclease and deoxyribonuclease was then added. The mixture was dialysed for 3 hr against 0.005 *M* tris buffer, pH 7.3, containing 0.01 *M* Mg^{++}, and centrifuged through a sucrose gradient in a 25 ml tube for 2.5 hr at 25,000 r.p.m. A pin hole was made at the bottom of the tube and drops were collected to get samples for which the optical density at 260 mμ (solid line) and the radioactivity (dotted line) were measured.

(3) *Chloromycetin*, at concentrations which inhibit protein synthesis in whole cells, was a strong inhibitor of the reaction (see Table 4).

(4) *Penicillin* (50 μg/ml), which stops the synthesis of cell wall proteins only, had no effect.

(*h*) *Analysis of amino acid incorporation in individual ribosomes:* The reaction mixture contained twice the amounts given in section a), with 5 μc labeled amino acids (25 μg C^{14} chlorella protein hydrolysate), in 0.01 *M* Mg^{++}. Two samples were run at 37°C for 45 min and 1 μg/ml DNase was then added. Sample 1 was dialyzed for 5 hr at 4°C against 0.005 *M* tris buffer with 0.01 *M* Mg^{++}, in order to remove free labeled amino acids, while sample 2 was dialyzed against 0.01 *M* phosphate buffer, pH 7.0, with only 0.0005 *M* Mg^{++}, a mixture in which 70 S ribosomes break down to 30 S and 50 S.[10] The samples were centrifuged at 5,000 *g* for 10 min and 1 ml of each of the supernatant fluids was layered on the top of a sucrose gradient in a centrifuge tube and centrifuged as indicated under methods.

The results obtained with sample 1 are illustrated in Figure 4. They show that the curve of radioactivity follows rather closely that of the optical density in the ultraviolet region: the labeled amino acids were about equally distributed among

the four classes of ribosomes. In Figure 5, the curve of the optical density for sample 2 shows that most of the 70 S and 100 S particles which were visible on the ultraviolet curve in Figure 4 have been broken down into 50 S and 30 S. However

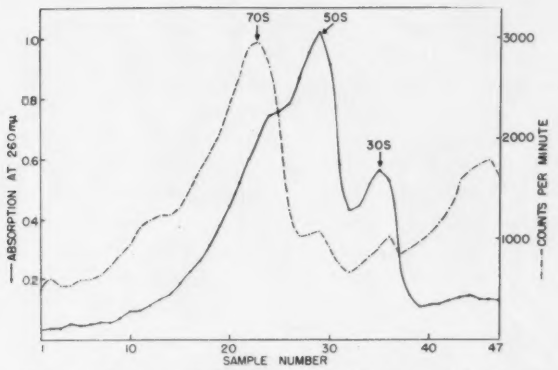

FIG. 5.—Incorporation of amino acids into *crude E. coli extract*. As in Fig. 4, with another aliquot of the same *crude extract*, except that dialysis was against 0.01 *M* phosphate buffer, pH 7.0, containing 0.0005 *M* Mg^{++}, for 5 hr, in order to break down 70 S ribosomes into 50 S and 30 S.

the curve representing the radioactivity does not follow the same pattern: most of the counts present in the 70 S and 100 S fractions in Figure 4 have remained with the 70 Speak (Fig. 5), and during the breakdown of 100 S and 70 S into 50 S and 30 S particles, very few counts, if any, have been transferred to the 50 S and 30 S fractions.

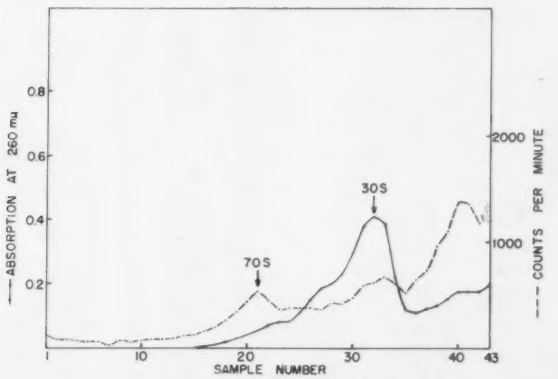

FIG. 6.—Amino acid incorporation into a 30 S ribosome preparation. Experimental conditions as in Fig. 4, with 2 mg of the 30 S fraction and 0.3 ml *supernatant*.

Thus a small proportion of the 70 S ribosomes originally present in the preparation, estimated at 5 to 10%, is highly labeled and is now stable to removal of magnesium. The specific activity of this stable 70 S fraction, which we shall call

"*active 70 S*" is indeed much higher than that of 30 S or 50 S: a sample containing *active 70 S* (sample 15 from Fig. 5) was examined with ultraviolet optics in the analytical centrifuge in order to get a rough estimate of the amount of *active 70 S* and the contribution of the contaminating 50 S particles to the optical density measurements. From the data of three experiments, we estimated the specific activity of the *active 70 S* to be 15 to 40 times that of the 30 S or 50 S.

(*i*) *Incorporation of amino acids by isolated 30 S and 50 S ribosomes:* These experiments were designed to answer the following questions: Do individual 30 S and 50 S particles incorporate amino acids? Can they form *active 70 S*?

Isolated 30 S and 50 S ribosomes were prepared as described under methods. They were tested for the incorporation of amino acids within 24 hr from the time the bacteria were harvested. The test mixture contained about 2 mg 30 S, or 5 mg 50 S ribosomes, or both, 0.3 ml supernatant and C^{14} labeled chlorella protein hydrolysate, with the other ingredients (as given in section *a*). The reaction was run at 37°C for 45 min. The mixture was then dialyzed for 5 hr against 0.01 *M*

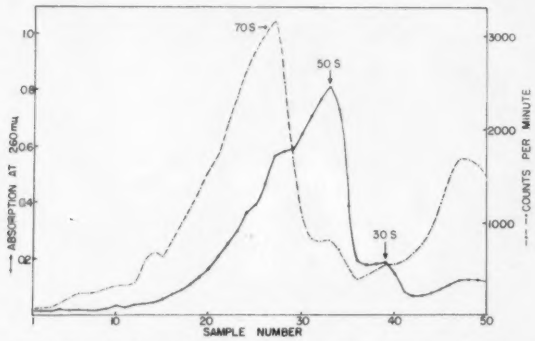

FIG. 7.—Amino acid incorporation into a 50 S ribosome preparation. Experimental conditions as in Fig. 4, with 5 mg of the 50 S fraction and 0.3 ml *supernatant*.

phosphate buffer, pH 7.0, containing 0.0005 *M* Mg^{++} to break inactive 70 S to 50 S and 30 S particles, and it was analyzed by the sucrose gradient technique as before. The results of a typical experiment with 30 S, 50 S, and 30 S + 50 S ribosome preparations are shown in Figures 6, 7, and 8, respectively.

It is clear that the contaminating *active 70 S* are largely responsible for the incorporation observed in the experiments; 30 S and 50 S particles incorporated very little by themselves. When they were both present in the reaction mixture, Figure 8 was obtained. It is very similar to the superimposition of Figure 6 on Figure 7: no *active 70 S* had been formed in the system.

Discussion.—The incorporation of amino acids was not due to the presence of bacterial cells, or large cell debris, as shown by the following evidence: (*a*) the incorporation did not take place in the absence of ATP and an ATP generating system, or at low Mg^{++} concentration; (*b*) the reaction rate slowed down with time and reached a plateau within 45 min; (*c*) the specific activity of the *crude extract* increased on removing small amounts of cell debris by 45 min centrifugation

at 20,000 *g*; (*d*) the *cell debris* fraction which, in view of the size of the debris, was the most likely to be contaminated by intact cells, had no activity.

Among the ribosomes present in the system described here, there is one special kind of 70 S particles, which we called *active 70 S*, the activity of which, in terms of amino acids incorporated, is 15 to 40 times higher than that of 30 S or 50 S ribosomes. The *active 70 S* form less than 10 per cent of the total number of ribosomes. They are present when the incorporation reaction starts, and are neither formed nor broken down in the system. Although there is evidence from time course and chasing experiments that a certain amount of labeled soluble proteins are released from the ribosomes, the *active 70 S*, after 45 to 60 min of reaction, are able neither to release nor to incorporate more amino acids. Only when new ribosomes are added does incorporation start again.

It seems therefore that during protein synthesis a factor, no longer operating in the *in vitro* system, should cause the *active 70 S* to release newly made proteins.

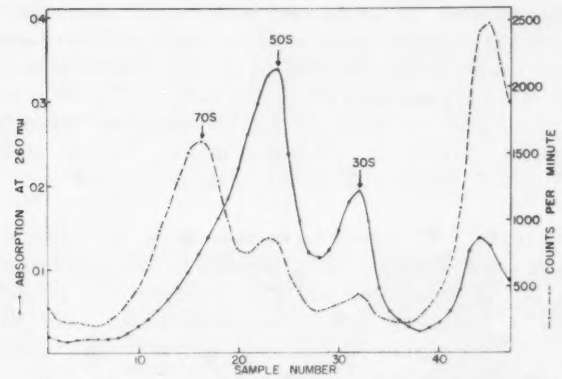

FIG. 8.—Amino acid incorporation into a mixture of a 30 S (2 mg) and a 50 S (5 mg) ribosome preparation and 0.3 ml *supernatant*. Experimental conditions as in Fig. 4.

The ribosomes would then be reactivated so that incorporation to form another protein molecule could again take place. A simple model would be that amino acids are incorporated in the *active 70 S*, in the groove between the 30 S and the 50 S particles; while the new protein molecule is released, the *active 70 S* splits to 30 S and 50 S, and then under the effect of an activator, 30 S and 50 S reform the *active 70 S*.

If we take the average value of 3.5 μg of amino acids incorporated during the reaction per mg of ribosomes, a 70 S particle of molecular weight 2.6×10^6 incorporated on the average a molecular weight of 9,100 or about 65 amino acids. Of this about $^2/_3$, or a molecular weight of 6,000, remained bound to ribosomes. Since the *active 70 S* are 15 to 40 times more highly labeled than the other ribosomes, they would each bear a molecular weight of newly made proteins of 46,000 to 122,000 or 380 to 1,000 amino acids.

In the first minute of the reaction, the incorporation reached approximately $^1/_4$ the level achieved in 45 min and the amount of amino acids incorporated corre-

sponded to a protein chain of molecular weight 2,280 per ribosome. On the other hand, in bacterial cells dividing every 20 min, ribosomes account for about 30 % of the dry weight[10] and protein for about 50%. If the ribosomes make all the proteins of the cell, 1 mg of ribosomes synthesizes about 100 μg of protein every minute. Therefore, in the first minute of the reaction, the cell free system is about 1% as efficient as the whole cell.

If, *in vivo*, the proteins are synthesized by the ribosomes, all present as *active 70 S*, the initial rate of incorporation of amino acids into *active 70 S* particles *in vitro* is of the same order of magnitude as the rate of protein synthesis in the cell.

Summary.—A cell-free system from *E. coli*, composed of ribosomes and soluble factors, incorporates amino acids with requirements similar to those of the liver system. The reaction rate falls with time and after about 4 min, half the total incorporation reached in 45 min is already achieved. The reaction is inhibited by ribonuclease, chloromycetin, and deoxyribonuclease.

About $^2/_3$ to $^3/_4$ of the labeled amino acids are bound to the ribosomes and $^1/_4$ to $^1/_3$ to the soluble proteins. A special kind of 70 S ribosomes, called *active 70 S*, incorporates 15 to 40 times more amino acids than 30 S and 50 S ribosomes. These *active 70 S*, which constitute less than 10% of the total number of ribosomes in the cell free extract, are neither formed nor broken down during the reaction.

The labeled amino acids can be chased from the *active 70 S* ribosomes mainly to the soluble proteins. DEAE chromatograms indicate that the amino acids are incorporated into most of the soluble proteins of the cell-free extracts.

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*THE COMPLETE AMINO ACID SEQUENCE OF THE PROTEIN OF TOBACCO MOSAIC VIRUS**

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Much evidence has accumulated in recent years to support the concept that the structure of proteins is subject to direct genetic control. The great advances in the methodology of protein structural analysis during the past decade have enabled Sanger, Tuppy, du Vigneaud, and others to conclude that corresponding proteins and polypeptides of different mammalian species usually differ by only one or two amino acid exchanges. Ingram found the same to be the case for the abnormal hemoglobins of man.

The simple viruses represent particularly suitable objects for the study of the genetic control of protein structure, since they appear to consist exclusively of the genetic material (nucleic acid) and the characteristic protein presumably produced as a result of the information carried by the nucleic acid. The protein portion of such a virus is suitable for analysis of its primary structure (amino acid sequence) since it consists of seemingly identical subunits of relatively low molecular weight. The structure of these protein subunits is of interest both because of its genetic implications and because its secondary and tertiary (folding) characteristics determine the way in which the protein subunits fulfill their biological function. This function is to aggregate in an ordered fashion around the viral nucleic acid thus forming the typical virus particles, a process which achieves marked stabilization and protection not only of the nucleic acid but also of the protein subunits themselves. The mechanism of this specific protein aggregation is probably akin to other protein interactions such as antibody-antigen and certain enzyme-substrate complexes. For the elucidation of this mechanism the primary structure of the protein must be known.

Tobacco mosaic virus (TMV), first to be recognized as a virus and since then often in the forefront of important advances in virology,[1–5] was chosen for structural analysis because it is the virus most easily obtainable in pure form and in great amounts, and because it has a large variety of distinctive strains which are also readily obtainable. Amino acid composition studies were started quite early, first by chemical isolation procedures[6–8] and colorimetric methods[9] and later by microbiological assay.[10] Until recently it seemed likely that the protein moiety

of TMV was a huge molecule having a molecular weight of many millions, although in weak alkaline solution this protein seemed to dissociate into units of molecular weight of about 100,000.[11] However, an opening wedge into the chemistry of the virus protein was the finding by Harris and Knight that carboxypeptidase split off from each virus particle (particle weight = 40×10^6) about 2,200 residues of threonine, and no other amino acids.[12, 13] If it was assumed that this represented the carboxyl-terminal (C-terminal) threonine of some 2,200 peptide chains, then their molecular weight could be calculated to be about 18,000, in agreement with the minimal molecular weight derived from cysteine analyses. The correctness of this supposition was supported by the results of hydrazinolysis experiments which also yielded one threonine per 18,000 molecular weight subunit.[14, 15] Soon thereafter the C-terminal amino acids of the enzymatically dethreoninated virus were shown to be -Pro-Ala and the sequence work was thus initiated.[16] The nature of the amino-terminal (N-terminal) groups, a subject of great perplexity, was resolved by Narita in this laboratory in 1958 when he demonstrated that the N-terminal serine was N-acetylated, a new type of peptide chain termination.[17]

Prior to this time a systematic study of the amino acid sequence of TMV protein had been initiated. Trypsin and chymotrypsin were used to split the protein in specific manner. The resultant peptides were first separated by paper electrophoresis and paper chromatography,[18] by column chromatography (Niu, unpublished, see reference 22), and later primarily by counter-current distribution, and analyzed for their amino acid composition and sequence. A series of publications from this laboratory reported on the number and end-group character of the tryptic peptides and described the composition of most, and the sequences of 7, of the 12 peptides.[19–23] Our approach to this work has been the cautious and critical one advocated by Hirs *et al.*[24] Thus, any sequence that could be more firmly established by the use of several different procedures was so tested and established. On the other hand, structural work by the group in Tübingen had first led these investigators to claim the existence of N-terminal proline, then of a β-aspartyl-proline loop. These and other reports concerning the composition of tryptic digests of TMV protein could not be substantiated and may be regarded as withdrawn.[25–30] However, during the past year the Tübingen group has made important contributions to the elucidation of the structure of the TMV protein. Thus, a complete sequence was proposed for the insoluble peptide (I-peptide) of 41 residues released by trypsin from the N-terminal position in the protein,[31] followed soon thereafter by a nearly complete structure for the virus protein.[32]

Our work has progressed to the point where we can now propose the first complete amino acid sequence for TMV protein (Fig. 1). In this proposed structure we confirm the sequential arrangement of tryptic peptides deduced by Anderer *et al.*[32] and by Wittmann[33] as well as many of their amino acid sequences. In addition we have been able to complete the sequences not previously worked out including location of 19 of the estimated 21 amide groups. The complete structure presented here contains a few sequences which differ from those previously published, and these points will be discussed in detail.

Results.—A. Sequential arrangement of tryptic peptides: To establish the sequential order of the twelve peptides obtained from the TMV protein by the action of trypsin, portions of protein were digested by subtilisin (Nagase) and by chymotryp-

sin (8 and 6 hr at 37°, respectively, at pH 7.8, with an enzyme substrate ratio of 1:100). The peptides containing basic residues (mostly arginine) were isolated by column chromatography (Dowex 1-X2, 150 cm × 2.8 cm column) using collidine-pyridine-acetic acid for development (pH gradient from 8.5 to about 3.0). The resultant peptide fractions were tested for purity by paper chromatography (pyridine, acetic acid, water, *n*-butanol), and further purified by this method and by electrophoresis, if necessary. They were then analyzed for their amino acid composition by means of an automatic analyzer.[34] Only the isolation of tripeptides or longer peptides carrying arginine in non-C-terminal position, or those containing a lysine which was not flanked by tryptophan and proline, was germane. That is, since peptides with such characteristics must possess sequences which overlap those of the peptides released by trypsin, they could be used to indicate which tryptic

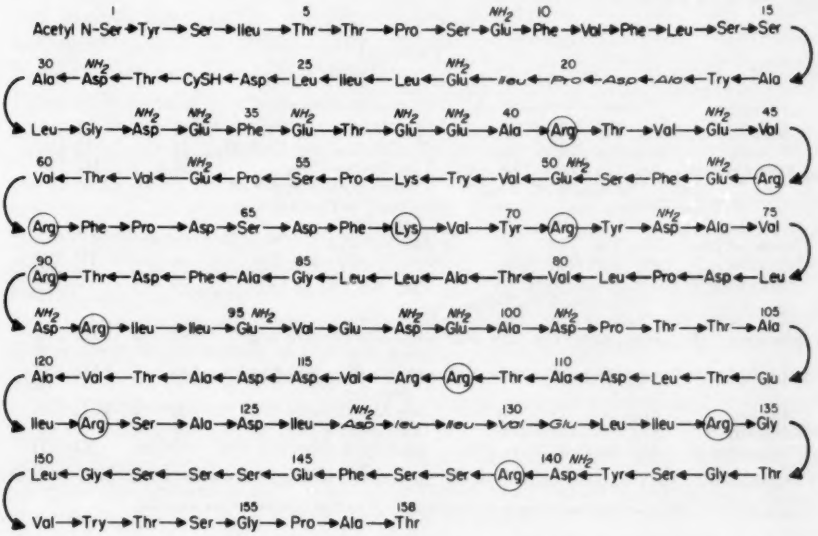

Fig. 1.—Sequence of the 158 amino acid residues in the protein subunit of tobacco mosaic virus. The encircled residues indicate the points of splitting by trypsin.

peptides were linked in the intact protein. Table 1 lists those peptides which fulfilled these requirements, and permitted a unique sequential arrangement of the twelve tryptic split products. Many more peptides were analyzed and none was found to be in conflict with the established amino acid sequence.

In previous publications from this laboratory the various peptides were identified by their K-value as found in the course of their isolation by counter-current distribution. The Tübingen group listed them in order of their separation by column chromatography. Since the sequence of the peptides is now well established, we propose to call them by their sequential number, starting with the N-acetylated I-peptide (No. I), and ending with the C-terminal threonine peptide (No. XII). Table 2 lists the peptides in this order, identifying each also by common laboratory designations and summarizing its composition.

B. Sequential arrangement of amino acids: A comparison of the complete sequence as given in Figure 1 with the nearly complete one published recently by Anderer *et al.*[32] reveals several differences which will now be discussed briefly. First, it must be noted that two sequences (#18–21, 127–131) are given in italics. This signifies sequences which are as yet under investigation and for which we have tentatively accepted those of the literature. Concerning the established part of the structure, it will be noted that in the present work the glutamine and asparagine residues have been distinguished from the corresponding acids. Since Ramachandran and Narita[35] estimated the total number of amide groups to be 21, and 19 have been accounted for, it appears possible that one or two additional amide groups may be located in those segments which are as yet under study. It should be noted that the relative number of glutamine and asparagine residues is not in accord with the results reported earlier.

TABLE 1

BRIDGE PEPTIDES* OF TMV PROTEIN

Enzyme	Amino acid composition	Postulated sequence†	Residue	Peptide
Subtilisin	Arg, Thr, $GluNH_2$, Ala	*$GluNH_2$*-Ala-Arg-Thr	#39–42	#I–II
Subtilisin	Arg, $(GluNH_2)_2$, Val	*$GluNH_2$*-Val-Arg-$GluNH_2$	44–47	II–III
Chymotrypsin	Arg, Lys, Asp_2, Thr, Ser_2, $GluNH_2$, Pro_3, Val_2, Phe_2	Lys-Pro-Ser-Pro-$GluNH_2$-Val-Thr-Val-Arg-Phe-Pro-Asp-Ser-Asp-*Phe*	53–67	III–IV
Chymotrypsin	Lys, Val, Tyr	*Lys*-Val-Tyr	68–70	IV–V
Subtilisin	Arg, Lys, Val, Tyr_2	Lys-Val-Tyr-Arg-Tyr	68–72	IV–V–VI
Chymotrypsin	Arg_4, Asp_5, Thr_5, Glu_4, Pro, Ala_3, Val_2, $Ileu_2$, Leu	*Asp*-(see Fig. 1)-*Val*	88–114	VI–VII–VIII–IX
Subtilisin	Arg_2, Asp_2, Thr, Ala, Val	*Asp*-Ala-Thr-Arg-Arg-Val-Asp	109–115	VIII-IX
Subtilisin	Arg_2, Asp_2, Thr, Ala, Val	*Ala*-Thr-Arg-Arg-Val-Asp-Asp	110–116	VIII–IX
Subtilisin	Arg_2, Asp, Thr, Ala	Asp-Ala-Thr-Arg-Arg	109–113	VIII–IX
Subtilisin	Arg, Gly, Ileu, Leu	*Leu*-Ileu-Arg-Gly	132–135	X–XI
Chymotrypsin	Arg, $AspNH_2$, Ser_2, Phe	*$AspNH_2$*-Arg-Ser-Ser-Phe	140–144	XI–XII

* Treatment of TMV protein with subtilisin and chymotrypsin yields peptides which overlap those released by trypsin and hence the term "bridge peptides."

† Residues in italics have been shown to be terminal by FDNB or hydrazinolysis methods.

TABLE 2

TRYPTIC PEPTIDES OF TMV PROTEIN

Sequential no.	Residue no.	Counter-current distribution nomenclature	Column nomenclature	Composition and terminal groups
I	1–41	I-peptide	XII	Acetyl-N-Ser (Asp_4, Thr_4, Ser_4, Glu_6, Pro_2, Gly, Ala_4, CySH, Val, $Ileu_2$, Leu_4, Tyr, Phe_2, Try) Arg
II	42–46	K-0.086	I	Thr (Glu, Val_2) Arg
III	47–61	K-0.66A	III	$GluNH_2$ (Thr, Ser_2, Glu_2, Pro_2, Val_2, Phe, Try, Lys) Arg
IV	62–68	K-0.33	IX	Phe (Asp_2, Ser, Pro, Phe) Lys
V	69–71	K-0.22D	V	Val-Tyr-Arg
VI	72–90	K-4.8	VIII	Tyr (Asp_3, Thr_2, Pro, Gly, Ala_2, Val_2, Leu_4, Phe) Arg
VII	91–92	K-0.065.2	II	$AspNH_2$-Arg
VIII	93–112	DCA-I-3	VII	Ileu (Asp_3, Thr_4, Glu_4, Pro, Ala_3, Val, Ileu, Leu) Arg
IX	113–122	K-0.13	VI	Arg (Asp_2, Thr, Ala_2, Val_2, Ileu) Arg
X	123–134	K-2.7	XI	Ser (Asp_2, Glu, Ala, Val, $Ileu_3$, Leu_2) Arg
XI	135–141	K-0.065.7	IV	Gly (Asp, Thr, Ser, Gly, Tyr) Arg
XII	142–158	C-terminal	X	Ser (Thr, Ser_3, Glu, Pro, Gly_2, Ala, Val, Leu, Phe, Try) Thr

Current studies on peptide I (the I-peptide)[36] suggest several differences from those of the literature, which require further substantiation. In regard to residue 24–30, in particular, it seems certain that the listed sequence (Ileu-Leu-Asp-CySH-Thr-AspNH_2-Ala-) and not that given by Anderer *et al.*[31] (Ileu-Asp-(CySH, Thr)-Asp-Leu-Ala-) is correct for the TMV with which we have worked. Details of this work on the I-peptide will soon be published by one of us (A. Tsugita).

The publication by Anderer *et al.* has confirmed our published sequences for peptides II, V, VII, and IX. However, in peptide III, they reported the sequence Pro-Ser whereas we first had reported Ser-Pro (#55, 56).[21] This part of the molecule was therefore reinvestigated by a method differing from that used by Gish previously.[21] Peptide III was digested with chymotrypsin and the expected lysine N-terminal fragment was subjected to the Edman degradation according to the procedure of Fraenkel-Conrat and Harris.[37] The first thiohydantoin released was that of ϵ-carbamyl-lysine, the second, that of proline, and the third, clearly and cleanly that of serine. The amino acid compositions of the residual peptides after step 1, 2, and 3 were Pro_2, Ser, Glu; Ser (Pro, Glu); and Pro, Glu. These data leave no doubt that our originally reported sequence of Ser-Pro as given on Figure 1 (#55, 56) is correct.

The sequence proposed for peptide IV by Anderer *et al.* is supported by some of our present data, while earlier data favored the original sequence.[20] The amino acid composition also suggests the presence of two aspartic acids in this peptide and the proposed complete structure incorporates, therefore, the sequence 65 to 68 of the Tübingen group. Peptide X is still under investigation; all sequential data obtained at present are in accord with the arrangement proposed in the literature. However, current studies on peptide VI indicate that there is a distinct difference in the sequence of residues #76 to 82, due to a change in the position of one leucine residue. Details on this work will be published by one of us (J. Young).

The complete structure for peptide VIII has been established and will be reported in detail by one of us (D. Gish). It must particularly be noted that this peptide contains one more amino acid than reported by Wittmann and Braunitzer[38] and by Anderer *et al.*[32] This is an isoleucine, which was probably missed in their work, because it occurs in the very acid resistant sequence of -Ileu-Ileu-. Its presence was established by 72-hour hydrolysis of a pentapeptide obtained from peptide VIII by subtilisin digestion; its composition was $Ileu_{(2)}$, $Glu_{(2)}$, Val. The sequence of #93–95 was established by the Edman method. This finding of an additional isoleucine is in accord with the amino acid composition of the protein as reported by Tsugita and Fraenkel-Conrat.[39] The data published by Wittmann[40] also support the higher isoleucine content (9 residues rather than 8 as concluded by Wittmann), and the total number of residues in the TMV protein is thus 158, and not 157 as indicated by Anderer *et al.*[32]

Our previously published sequence for peptide XI[20] reported Ser-Gly at positions 137–138 (Fig. 1), whereas Anderer *et al.*[32] found the inverse, Gly-Ser. Our previous conclusion was based on the relative rate of release of serine and glycine upon digestion of the peptide with aminopeptidase. More recently, peptide XI was digested with chymotrypsin, and the product Gly (Gly, Thr, Ser, Tyr) was subjected to carboxypeptidase action which caused release of the tyrosine. The remaining peptide was then treated with hydrazine. Only serine was detected,

thus supporting the Gly-Ser sequence of Anderer *et al.* for residues 137 and 138. A technical improvement of the hydrazinolysis procedure was used in that the evaporated hydrazinolysis mixture, treated with isovaleraldehyde if necessary, was applied directly to the amino acid analyzer thus permitting the easy identification and determination of the C-terminal amino acid, the hydrazides of most other residues remaining bound to the column.

The amino acid sequence of peptide XII has in part been previously published[41] and *in toto* referred to[39] and will be reported in detail by one of us (D. Gish). It was confirmed by the studies of the Tübingen group.

Discussion.—TMV protein has become the first viral protein with a completely determined amino acid sequence. It is also the largest protein to have been completely elucidated inasmuch as it contains 158 amino acid residues, whereas the other two proteins whose primary structures have been established are insulin and ribonuclease with 51 and 124 amino acid residues, respectively.

The great extent of the agreement in sequences proposed by the groups at Tübingen and at Berkeley, after independent investigations, lends confidence in the validity of the results. The conformity of results supports the long-standing assumption that TMV is a definite chemical entity. Such differences as remain may well be due to analytical technicalities which will eventually be resolved. However, there is an alternative possibility which cannot presently be excluded—that the two groups have analyzed slightly different strains of TMV.

In any case, the base has now been established for a variety of studies involving chemical structure and function. By applying the same techniques to naturally occurring strains or to strains arising from various chemical or physical treatments, it will be possible to trace the effect of mutation on viral protein in an unprecedented, detailed fashion. For example, important differences in the C-terminal sequence of TMV protein and the naturally occurring strain, HR, and between TMV protein and a nitrous acid mutant have already been demonstrated.[16, 39] The latter represents the first case in which mutation in the nucleic acid was caused by a known laboratory reaction with a concomitant change in the protein subsequently produced, which change has been located at a specific site of a fully known protein sequence. The next step will be to relate the structure of viral nucleic acid to its specific protein in a point-to-point manner. Although progress is also being made in this direction, it will probably be some time before the methodology of nucleic acid chemistry will catch up with that of protein chemistry and the code relating one structure to the other will have become unraveled. This is, of course, the problem of greatest importance in biology and medicine, and it appears that the viruses will provide a unique experimental approach.

Knowledge of the complete primary structure of TMV protein, when coupled with the currently known structural features of the TMV particle, also provides the basis for a still more detailed three-dimensional model of the virus. From this there should come a better understanding of the tertiary and quaternary structure of the virus particle, the chemical basis of its serological specificity, its reactivity with enzymes, and its participation in the metabolism of the infected cell.

Summary.—On the basis of new and previously published data, a complete sequential arrangement of the 158 amino acid residues of the protein subunit of tobacco mosaic virus is reported.

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*EXCITATION ENERGY TRANSFER AND THE QUANTITATIVE STUDY OF THE ANTIBODY HAPTEN REACTION**

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When certain kinds of organic molecules become attached to proteins, either as biochemically specific complexes or as synthetic chemical conjugates, the quantum yield of the fluorescence of the tryptophane residues of the proteins is diminished. A condition for such an effect is the overlap of an absorption band of the ligand with the emission band of the protein. If the spectral condition and a few geometric requirements are satisfied the excitation energy of the tryptophane residues may be transferred with high efficiency to the ligand by electric dipole-dipole interactions over distances the order of magnitude of a protein molecular diameter. The excitation energy in the ligand may then either be dissipated as heat or reemitted at the characteristic frequency of the ligand. This and related phenomena were utilized previously in an analysis of the complexes formed between pyridine and flavin nucleotides and the specific proteins for which they serve as coenzymes.[1, 2] The present report deals with the occurrence and analysis of excitation energy transfer in the complexes between purified antibodies and the small organic molecules used as determinants in their induction. Two antibodies were employed, directed respectively against derivatives of 2,4-dinitrophenyl and against derivatives of *p*-azophenylarsonate. Such molecules have strong absorption bands in the 300 to 400 mμ region which overlap the 350 mμ emission bands of the antibodies. Formation of soluble complexes between antibody and univalent hapten is quantitatively measurable with the quenching of the antibody fluorescence as the indicator. The unique sensitivity of fluorescence spectroscopy makes possible the direct and rapid measurement of the antibody-hapten reaction over a range of conditions not approachable by other methods.

Methods.—Induction and purification of antibodies: The antibodies were induced in rabbits by single injections, with Freund's adjuvant, of 5 mg of bovine γ globulin substituted on the ε-amino groups of the lysine side chains with 2,4-dinitrophenyl groups, or on the lysine, histidine, and tyrosine side chains with azobenzene-*p*-arsonate. The dinitrophenyl antigen carried 55 moles of hapten per mole of protein, by spectrophotometric analysis of the intact antigen and by silica gel chromatography of acid hydrolysates. Antibodies from the pooled high titre sera of 20 rabbits were isolated by specific precipitation with antigen.

The immunizing antigen was also used to precipitate the anti-DNP antibody. Because of the large number of substituent groups only a small amount of antibody was produced against the protein itself. Antibody specific for the hapten determinant was separated from the precipitated complex by stirring at pH 7.4. with 0.1 *M* 2,4-dinitrophenol in the presence of streptomycin. The univalent hapten competes with the antigen to form a soluble antibody complex and the acidic test antigen is kept in the solid phase by the formation of a streptomycin salt. Antibody and hapten were separated from each other by column chromatography on

Dowex-1, ammonium sulfate precipitation, and dialysis. The antibody so purified was 90 to 95 per cent precipitable by the antigen. No non-γ-globulin impurities could be detected by sedimentation or electrophoresis. Concentrations of test solutions were determined spectrophotometrically using a specific absorption coefficient of 1.46 cm^2/mg based upon dry weight. Details of the purification are described elsewhere.[3]

The antibody to the p-azophenylarsonate determinant was precipitated from the pooled antisera with an azophenylarsonate derivative of bovine-γ-globulin. In this case, in order to render the precipitating antigen less soluble at acid pH, the free amino groups of precipitating antigen were blocked with 2,4-dinitrophenyl. The antibody complex, in contrast with that of the anti-DNP system, dissociated at pH 3.8 without the use of a competitive hapten and the precipitating antigen was again retained in the solid phase as the streptomycin salt. Antibody obtained by this method was 82 per cent precipitable with test antigen.

Preparation of haptens: 2,4-dinitrophenol and α-2,4-dinitrophenylacetic acid were recrystallized from water, mp 113 and 186–187 (uncorr) respectively. Previously described procedures were used to prepare ϵ-N-DNP-lysine and ϵ-N-DNP-amino-caproic acid.[4] O-mono-DNP tyrosine and α-ϵ-bis-DNP-lysine were obtained from Mann Research Laboratories, New York City. *p*-(*p*-hydroxyphenyl-azo)-phenylarsonic acid was made by coupling an excess of diazotized *p*-arsanilic acid with phenol. After purification by repeated precipitation at pH 3 the millimolar absorption coefficient was 25.4 at 435.5 mμ in 0.1 *N* Na*OH*. Mono and bis *p*-phenylarsonate azotyrosine were synthesized and kindly supplied by Dr. M. Tabachnick.[5] Bovine-γ-globulin (BγG) was substituted, almost maximally, with DNP groups by reaction with 2,4-dinitrobenzene sulfonate[6] (Eastman Kodak; recrystallized 4 times from water). Azobenzene arsonate groups (R-azo) were introduced into proteins by coupling with about 200-fold molar excess of diazotized *p*-arsanilic acid, pH 9. The number of hapten groups introduced by this method is much less than with the DNP antigen. The R-azo-precipitating antigen used for isolation of antibody was prepared by treating maximally substituted DNP-bovine-γ-globulin with diazotized arsanilic acid, as above. In general substituted proteins were purified at 4° by repeated precipitation and washing at pH 4, followed by prolonged dialysis and passage through 15 × 1 cm ion exchange columns of IRA-400, 3–5X, 20–50 mesh, chloride form.

Fluorescence measurements: A commercial instrument (Aminco), employing two grating monochromators at right angles to the sample, was employed. The light source was replaced by a stable dc operated high pressure xenon arc (Osram XBO 162) and a water jacket was built around the sample holder for temperature control. High spectral resolution is not essential but a stable light source and temperature control are important. Aliquots of the stock antibody solutions were transferred to the desired buffer, 1.0 to 2.0 ml final volume, in a 1 cm square quartz cuvette. Fluorescence of the protein was excited usually at 290 mμ where protein absorption is strong and absorption by the hapten is minimal. The protein emission at 350 mμ was brought on scale by adjusting the gain of the detector and was read in arbitrary intensity units on a microammeter. Aliquots of the hapten solution were then added from a micropipette and the protein emission intensity was read after each addition and corrected for the small amounts of dilution. At low con-

centrations free hapten has no effect on the protein emission, but complex formation results in strong quenching. At higher concentrations free hapten may diminish the protein emission, by attenuation of the excitation beam. The latter effect is readily detected and the attenuation when it occurs may either be corrected for, or avoided by, modification of the geometry of the test system. In a few cases the hapten was added continuously to the stirred test solution from a microsyringe driven by a synchronous motor and the fluorescence changes were measured automatically on a strip chart recorder. In the latter case the total volume of the titrant, added over a 6-min period, was less than 0.04 ml.

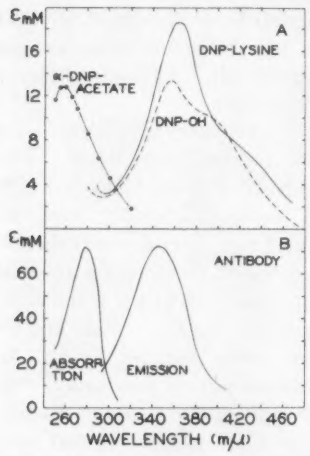

FIG. 1.—A-Absorption spectra of the 2,4-dinitrophenyl haptens. B-Absorption and emission bands of the antibody and its proteolytic fragments (see text). Millimolar absorption coefficients in this case are those of the proteolytic fragment II. The height of the emission band maximum is by convention set equal to that of the absorption maximum although the fluorescence quantum yield is less than 20 per cent.

Results.—Absorption and emission spectra: The absorption spectra of the dinitrophenyl derivatives used as haptens and the absorption and emission bands of the antibody are shown in Figure 1. The antibody used for the spectrum in Figure 1 is the active proteolytic fragment II discussed later in the text. The spectra of the proteolytic fragments and of the intact antibody are quite similar in band shape and position, and in fluorescence quantum yield. Fractions I and II and the intact antibody have nearly the same specific absorption coefficient at 280 mμ and the absorption coefficient of the crystalline fraction III is about 30 per cent lower. There is extensive overlap in the absorption bands of the DNP-lysine and phenol derivatives with the emission band of the protein thus satisfying the condition for efficient electrical dipole interaction in the excited state. The overlap of the protein emission band with the absorption band of the α-DNP-acetic acid is smaller and results as will be seen later in a weaker interaction. The pertinent absorption bands of the 3-(*p*-azophenylarsonate)-tyrosine and 4-(*p*-azophenylarsonate)-phenol are maximal at 325 and 360 mμ, with millimolar absorption coefficients of 22 and 20, respectively. Thus these compounds also satisfy the spectral condition for the quenching of the protein fluorescence.

Stoichiometry: Figure 2 shows the decline in the fluorescence of the antibody when it is titrated with the hapten, ε-N-DNP-lysine. The antibody emission is quenched about 75 per cent and the endpoint is clearly discernible by extrapolation of the linear initial portion of the curve to the terminal base line. From numerous titrations of this type the equivalent combining weight of the purified antibody is 98,000 gm protein per mole of hapten. Since the antibody is only 90 to 95 per cent precipitable the equivalent weight of the active antibody may be as low as 88,000 gm. Molecular weights of rabbit γ-globulin have been reported in the range 160,000 to 188,000 and a value close to 160,000 was obtained by the sedimentation diffusion method with the present material. The titrations indicate, with minimal

correction and extrapolation, 1.8 binding sites per molecule of antibody (MW 160,000), thus confirming the value of 2, arrived at over a period of years, by several workers.

Dissociation constants: Titrations of the type shown in Figure 2 for optimal determination of stoichiometry are carried out at antibody concentrations of the order of 0.2 mg per ml. When the titrations are performed at 10 to 20-fold higher dilution the dissociation of the complex is more easily measurable (Fig. 3). The dissociation constant is remarkably small, of the order of 10^{-9} M. Since the combining capacity of the sample is known, the intrinsic dissociation constants may be calculated for individual points. Experimental results are then compared with theoretical curves for intrinsic dissociation constants in the range observed. A set of theoretical curves in the range K = 10^{-8} to 10^{-9} M is shown in Figure 4 for antibody at a concentration of 0.05 micromoles per liter (0.1 microequivalents of active site per liter). The results are reproducible within a factor of 2 in K or ± 0.3 kcal in $\Delta F°$, the standard free energy of dissociation. Since $\Delta F°$ in this case is 11.3 kcal per mole the error in $\Delta F°$ is approximately 4 per cent.

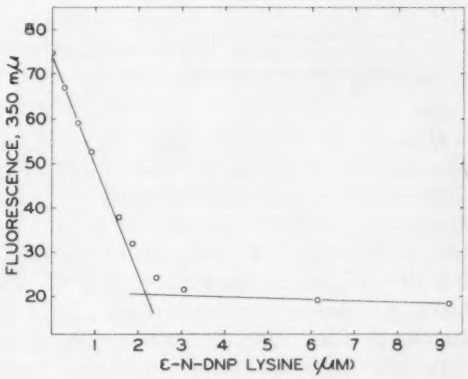

FIG. 2.—The titration of 0.21 mg of antibody with ε-N-DNP-lysine at 26 degrees, pH 7.4 in 1.0 ml of 0.01 M potassium phosphate buffer 0.15 M sodium chloride. The intersection of the linear extrapolations is the antibody concentration, microequivalents per liter.

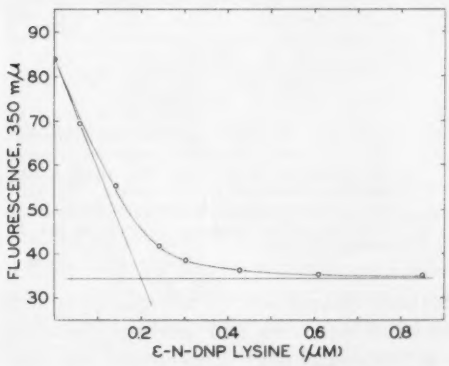

FIG. 3.—The titration of 0.02 mg of antibody under the conditions of Fig. 2. The apparent decrease in quenching efficiency, compared to Fig. 2 arises chiefly from an increased instrumental blank at the required higher gain of the detector. Similar titration curves are obtained with E-N-DNP-amino caproate.

Table 1 summarizes the results of some dialysis equilibrium measurements that were set up on the assumption that the dissociation constant of the complex was about $10^{-5}M$, in the range of values commonly reported for other antibody hapten systems. At a concentration of 35 micromolar the antibody within the dialysis sac adsorbed 55 micromolar hapten from the outer solution leaving no significantly detectable free hapten. The strong binding indicated by the fluorometric titrations is thus confirmed by dialysis and it is clear that the dialysis method is not suitable for the determination of such small dissociation constants.

TABLE 1

DIALYSIS EQUILIBRIUM OF ANTIBODY VERSUS ε-N-DNP-LYSINE

Hapten concentration, micromolar

Exp. initial conditions	Outer Solution		Inner Solution	
	Initial	Final	Initial	Final
1. No antibody	55.0	27.1	0.0	27.1
2. Antibody in inner solution, 5.65 mg per ml	0.0	<0.06	55.0	55.0
3. Antibody in inner solution, 5.65 mg per ml	55.0	<0.06	0.0	55.0

Homogeneity of binding sites: The experimental points of the fluorometric titrations fall, within the limits of experimental error, on the theoretical curves for single intrinsic dissociation constants. The accuracy of the average K's has been discussed. Limits of heterogeneity are more difficult to establish. It is not possible, for example, to distinguish by the present methods between a single K of 5×10^{-9} M and an equimolar mixture of two K's of 10^{-8} and 10^{-9} M. Within these limits, in titrations over the pH range, 1.5 to 8, the purified antibody appears to be homogeneous in the region of 15 to 85 per cent saturation. The method of purification makes it unlikely that an appreciable amount of more strongly binding species is concealed in the first 15 per cent to be titrated. It is possible that a small fraction of the antibody molecule population exhibits a larger than average hapten dissociation

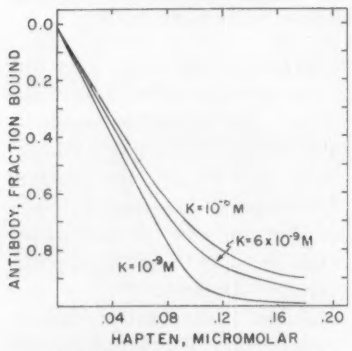

FIG. 4.—Theoretical hapten titration curves for 0.1 microequivalents of antibody per liter.

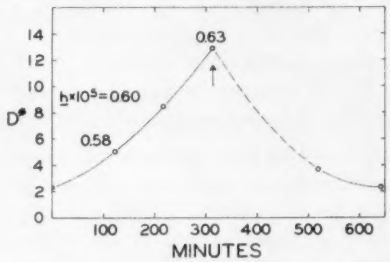

FIG. 5.—The apparent diffusion constant, D*, of the anti-DNP-antibody during electrophoresis at pH 7.3. The arrow marks the time of reversal of the current. The buffer in this experiment was 0.02 M trishydroxymethyl amino methane chloride in 0.1 M sodium chloride.

constant. Thus at extremely high dilutions of antibody, at approximately 0.05 microequivalents per liter, the apparent stoichiometries and maximal quenching may be about 5 per cent less than those obtained in more concentrated solutions. There is some evidence that antibody hapten dissociation constants, and perhaps homogeneity, are affected, in the anti-DNP system, by the method of immunization. This is being investigated.

Electrical charge inhomogeneity: When proteins are subjected to free electrophoresis at low current densities at or near the isoelectric point the boundaries broaden more rapidly than in free diffusion and the excess spreading may be reversed by reversing the current. Alberty and co-workers[7] observed this effect with several proteins that satisfy the ordinary criteria of purity but the effect was

unusually pronounced with γ-globulin. Since the boundaries retain the form of a normal distribution curve the results are accounted for in terms of a Gaussian distribution of mobilities within the molecular population. It was suggested that the differences in folding of the various specific antibodies in a general γ-globulin preparation might be associated with shifts in the *pK*'s of acidic groups sufficiently large to give a spread of several charge units in the population of molecules at a given pH. Purified antibody, specific for a given determinant, might therefore be expected to exhibit a much smaller degree of reversible boundary spreading. Electrophoresis and diffusion experiments were accordingly performed on the purified anti-DNP-γ-globulin, 90 to 95 per cent of which reacted uniformly with a single hapten. The results are shown in Figure 5 where the apparent diffusion constant, D^*, is plotted as a function of time of electrophoresis. D^* increases continuously with time over a 5 hr period as a result of electrical inhomogeneities and declines to the normal value for free diffusion when the current is reversed for an equal

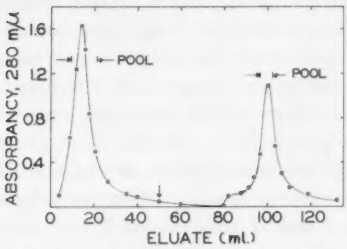

FIG. 6.—Chromatography of proteolytic fragments I and II of anti-DNP-γ-globulin after removal of most of fragment III by crystallization. The column was 20 × 1.1 cm carboxymethyl cellulose. The first peak was eluted with 0.01 *M* sodium acetate, pH 5.5. At the arrow a gradient was initiated in which 0.9 *M* sodium acetate was added to a mixing chamber containing 250 ml. of 0.01 *M* sodium acetate, pH 5.5.

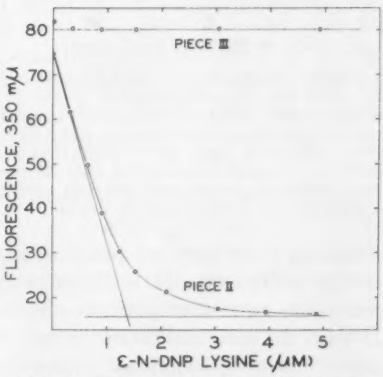

FIG. 7.—Titration of proteolytic fragments II and III, 0.105 and 0.247 mg respectively, in 1.0 ml of 0.1 *M* sodium acetate buffer, pH 4.1.

period of time. Reversible spreading is described in terms of a heterogeneity constant, *h*, the standard deviation of the Gaussian mobility distribution. The values obtained by Alberty *et al.* varied from 0.5 to 0.8 in contrast to values of 0.2 for enzymes. In the present experiments *h* falls in the range 0.58 to 0.63 and is thus close to the value obtained with general γ-globulin. Contrary to Alberty's hypothesis the charge heterogeneity appears to be independent of the nature of the binding site. The possibility must be considered that there are actual differences in amino acid composition or sequence within the globulin population. Alternately the heterogeneity may still arise from a heterogeneity of *pK*'s due to variations in secondary or tertiary structure and these may occur in regions of the antibody that are not relevant or are only partially involved in the actual binding sites.

Proteolysis of the antibody: The purified anti-DNP-γ-globulin was treated with papain and then fractionated under the conditions recently described by Porter[8] for the unresolved γ-globulin mixture. As described by Porter a crystalline degradation product was obtained in good yield and the more solublefr actions were

separated chromatographically on a carboxymethyl cellulose column (Fig. 6). The molecular weights of soluble fractions I and II are 50,000. Porter concluded that they contained univalent antibody fragments because they inhibited the precipitin reactions exhibited by antibodies present in the intact γ-globulin. This interpretation is confirmed by direct fluorometric titration of the pieces from the purified antibody (Fig. 7).[9] The fluorescence of the crystalline fragment III is not quenched by hapten and hence the hapten is not bound, in accord with the absence of precipitin inhibition. Fraction II titrates fluorometrically like intact antibody. The minimal combining weight is 75,000 gm per mole of ε-N-DNP-lysine. Titrations of the total proteolysis mixture before and after papain action had indicated that about 15 per cent of the combining capacity of the antibody was lost during proteolysis. If the inactivated material accompanied fractions I and II during chromatography then we would expect I and II to be about 75 per cent active since the starting material contained up to 10 per cent inactive γ-globulin. The equivalent combining weight of the active piece II is therefore approximately $^3/_4 \times 75{,}000 = 55{,}000$ gm in reasonable agreement with the observed molecular weight. Piece I titrates like II with the same stoichiometry but tails off more gradually as saturation is approached. This fraction shows binding heterogeneity which we must attribute to modifications resulting from proteolysis.

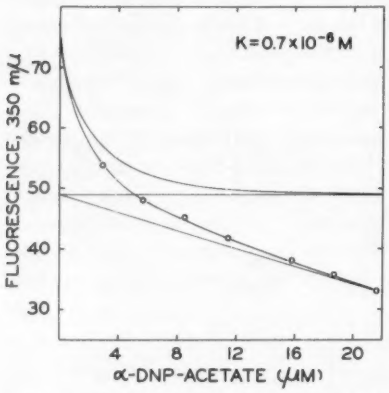

FIG. 8.—The binding of γ-DNP-acetic acid by antibody. Direct absorption of the excitation beam by hapten is responsible for the the terminal slope, corrections for which have been made in the upper curve. Note that the antibody emission is quenched only 35 per cent because of poor overlap between hapten absorption and protein emission bands.

Variation of hapten structure: Of the various DNP derivatives that might be tested the ε-N-DNP-lysine would be expected to show the maximal affinity for antibody because the majority of the DNP groups in the antigen were coupled to lysine side chains. Experiments with 2,4-dinitrophenylacetic acid and 2,4-dinitrophenol are shown in Figures 8 and 9. Although both of these derivatives interact strongly with antibody the dissociation constants are larger than those for the lysine derivatives by 2 to 3 orders of magnitude. Similar results, not shown, were obtained with O-DNP-L-tyrosine. It was therefore necessary to

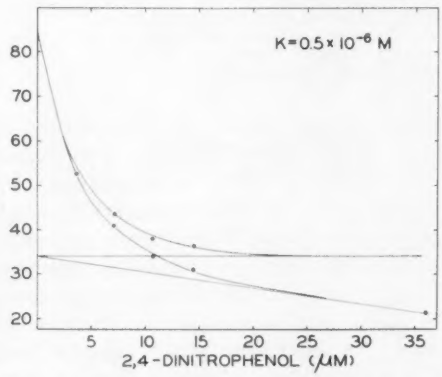

FIG. 9.—Fluorometric titration of antibody with 2,4-dinitrophenol at pH 7.4, 26°.

work with more concentrated solutions and as a result there was attenuation by direct absorption in the hapten. This resulted in a diminution of protein fluorescence that was independent of energy transfer within the complex. The terminal slopes of the titration curves are therefore not horizontal. Corrections for attenuation are provided by extrapolating the terminal slope back to zero hapten concentration. At any experimental hapten concentration the fluorescence correction is the difference between the corresponding point on the terminal slope line and the intercept. Corrected curves so obtained show good agreement with theoretical curves for a single intrinsic dissociation constant and equivalent binding sites. The validity of the correction is established by substituting for the antibody any protein which has no significant affinity for the hapten. The fluorometric titration curve is then linear from the start with the same slope as the terminal slope of the antibody titration. The dissociation constants of the antibody complexes of the acetic acid and phenol derivatives are respectively 0.75 and 0.5×10^{-6} M. The larger value of the dissociation constant is the reason for using the phenol instead of the lysine derivative for resolution of the antigen antibody complex in the original isolation of the antibody. At the concentrations employed the phenol dissociates about half of the antibody from the precipitate and may be subsequently removed from the soluble complex. The lysine derivative because of its higher antibody affinity dissolves virtually all of the antibody from the antigen complex but methods have not yet been found for removing this hapten from the soluble complex in good yield.

The behavior of divalent hapten: When antibody at a concentration of 1 μN was titrated with the divalent hapten, α,ϵ-N-di-DNP lysine, the quenching of protein fluorescence was linear with hapten concentration up to the point of one mole of hapten per two moles of antibody. In the vicinity of this point, and beyond, turbidity began to develop. Thus, because of the high affinities in the anti-DNP system stable linear aggregates are formed of a size sufficiently large to precipitate from very dilute solution. Epstein[10] and co-workers, dealing with a more dissociable type of divalent hapten antibody system observed the formation only of small soluble chains by the light scattering method.

Protein-protein interactions: The combination of antibodies with unmodified protein antigens is not susceptible to fluorometric analysis by the present method because (*a*) the reaction usually results in precipitation and (*b*) there is no fluorescence quenching interaction. Both of these difficulties may be circumvented with the anti-DNP system. The fluorescence of the DNP antigen is completely suppressed by the hapten groups and these serve also to quench the fluorescence of the antibody in the antibody antigen complex. Precipitation may be avoided by using the univalent proteolytic cleavage fragments of the antibody. The results of a titration of purified proteolytic fragment II of anti-DNP-antibody with bovine-γ-globulin that carries 55 moles of DNP per mole of protein are shown in Figure 10. Piece II in this case had been stored in solution for one month and then dried in the frozen state. After this treatment the dissociation constant with ϵ-N-DNP-lysine had increased to 2×10^{-7} M but there was no change in the stoichiometry. A sample of the univalent antibody that carried 1.3 millimicromoles of ϵ-N-DNP-lysine binding sites gave an end point with an amount of intact antigen that carried 4 millimicromoles of bound hapten. Thus about one third of the hapten groups

on the antigen are available for combination with the antibody at any one time. The limiting factors are the local environments of the hapten groups on the antigen and the number of antibody fragments that can be packed on the antigen surface. Problems of structural and statistical interest in connection with the protein-protein complex cannot be dealt with properly until the antigen, which is by no means molecularly homogeneous, is more fully characterized. If one expresses antigen concentration in terms of the limiting number of available hapten groups the titration curve of antibody with antigen is virtually superimpossible on the curve obtained by titration with free ϵ-N-DNP-lysine. The interaction of antibody with antigen-bound hapten is thus comparable in strength with the interaction with free hapten.

The effects of pH and urea: The apparent dissociation constants of the ϵ-N-DNP-lysine complex as a function of pH at 31° are summarized in Table 2. Combination of hapten with antibody is relatively insensitive to hydrogen ion con-

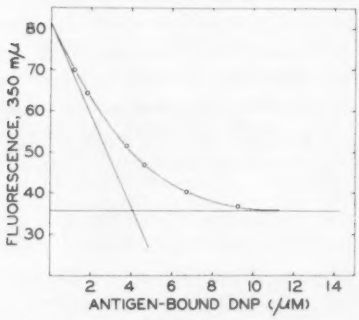

FIG. 10.—Protein-protein interaction. A sample of univalent anti-DNP fragment II, corresponding to 1.3 millimicromoles of ϵ-N-DNP-lysine binding sites, is titrated with intact antigen. The abcissa is the micromolar concentration of the total antigen-bound hapten groups. The titration curve has been corrected for attenuation.

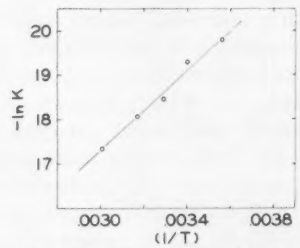

FIG. 11.—Plot for the determination of $\Delta H°$, the standard enthalpy change in the formation of the ϵ-N-DNP-lysine complex with antibody.

centration over the broad pH range 3 to 9. On the extreme acid side, pH 1.5, the increase in K is associated both with a rapid and a slow change in the protein and is not completely reversible if the protein is maintained at the low pH. The increase in K in the alkaline pH region is much greater than in pH 1.5 and corresponds to a protolytic pK somewhere in the pH region 10 to 11. The changes in this pH region are reversible.

TABLE 2

THE DISSOCIATION CONSTANTS OF THE ϵN-DNP-LYSINE ANTIBODY COMPLEX AS A FUNCTION OF pH AT 31°

pH	1.5	2.	3	4.	7.3	8.5	9	9.5	10.	11
−log K	7.	7.5	8	8.1	8.1	8.1	8	7.7	7.4	<5

The stability of the protein is illustrated by its behavior in concentrated urea solution. After a 12-hr incubation of the antibody in 7 M urea at 5° the stoichiometry of hapten binding is essentially unaltered and the dissociation constant of the

ϵ-N-DNP-lysine complex in 7 M urea, pH 7, is less than 10^{-6} M. The binding is also insensitive to changes in ionic strength from 0.01 to 1.0.

Thermodynamic constants: The antibody-hapten reaction has been studied over the temperature range 4 to 60°. Although the antibody is not stable indefinitely at the higher temperatures it takes only a few minutes to make equilibrium measurements by the fluorometric method and during such intervals the temperature dependent changes in the protein are completely reversible. The standard free energy change of complex formation, $\Delta F°$, is constant at -11.3 kcal mole^{-1}. From the plot $-\ln K$ versus $1/T$ (Fig. 11) the standard enthalpy change for complex formation is found to be $\Delta H° = -8.6$ kcal mole^{-1}. In accord with the results of Karush[11] on an azobenzene hapten antibody system the enthalpy and not the entropy factors are dominant in the strong binding. The standard entropy change in the present case is 9 eu.

The azobenzenearsonate antibodies: Whereas the hapten groups in the DNP antigens are predominantly on the ϵ-HN_2 groups of the lysine side chains, the azobenzene haptens are distributed in a more varied manner, occurring in the carrier protein as mono and bis R-azotyrosine, R-azo histidine, and R-azo lysine. Mono and bis azophenylarsonate derivatives of tyrosine were employed as test haptens. The divalent hapten, bis-R-azo-tyrosine, as in the case of the divalent hapten in the DNP series, caused precipitation of the antibody. The mono substituted tyrosine derivative yielded the titration curve shown in Figure 12. In this case the dissociation constant is 1×10^{-6} M and the stoichiometry corresponds to about 75 per cent active protein. Quantitative precipitin tests indicated about 80 per cent active antibody on a protein weight basis. There are no gross signs of heterogeneity. If the nature of the side chain to which the determinant group is coupled in the antigen influences the specificity this antibody should show distinct signs of heterogeneity when examined in greater detail.

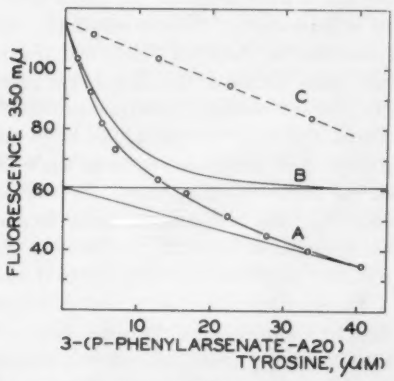

FIG. 12.—The combination of mono-p-phenylarsonate-azo-tyrosine with the anti-azophenylarsonate antibody. Curve A contains the observed points, curve B is corrected for attenuation, and curve C is a control in which the antibody titrated is anti-DNP instead of anti azophenyl arsonate. No binding of the azo hapten occurs in the latter case, the slope arising entirely from attenuation of the excitation beam.

Domains of interaction: As in the case of the pyridine nucleotide enzyme complexes[2] each molecule of ligand in the antibody-hapten system quenches an equivalent fraction of the protein fluorescence and the effects are additive. A molecule of bound hapten thus interacts only with the tryptophane residues within its own domain and the two domains do not overlap. There are 22 tryptophane residues in a molecule of rabbit γ-globulin of molecular weight 160,000. The fluorescence quantum yield of the protein is about 10 per cent, but since a third or more of the excitation energy is absorbed and dissipated thermally by the tyrosine residues, the fluorescent quantum yield of the constituent tryptophane is closer to 20 per cent,

the value exhibited by free tryptophane. It is thus likely that all of the tryptophane residues contribute to the fluorescence. Maximal quenching by two molecules of bound hapten is of the order of 70 per cent and may be as high as 80 per cent if we correct for the presence of inactive protein. Each molecule of hapten thus captures in effect all of the excitation energy from $0.8 \times 22/2 = 8.8$ tryptophane residues. Judging from the absorption spectra, pieces I and II contain approximately 8 tryptophane residues each and III contains 6. A quenching domain thus appears to correspond roughly with the fragments that are cleaved by papain. That this is true is indicated by the fact that the small decrease in the extent of quenching in the papain digest is accounted for largely by destruction of some of the binding sites. If quenching extended appreciably into the inactive piece III the change in the quenching after proteolysis would exceed the change in stoichiometry. Correspondingly the pure pieces I and II should be totally quenched by hapten. Actually the observed quenching is about 65 per cent and when corrected for 25 per cent inactive material it is 87 per cent. In terms of the coupled oscillator mechanism the effective interaction distance between tryptophane and DNP lysine should be of the order of 50 A and it is therefore not surprising that about one-third of the elongated γ-globulin molecule be out of range of the hapten binding sites. It is coincidental however that proteolysis occurs so near the lines of demarcation of domains unless the domains are limited not only by interaction distances but also by structural discontinuities or areas of relative disorder that are more susceptible than average to proteolytic attack.

Discussion.—The method of measuring soluble antigen-antibody and antibody-hapten complexes by the quenching of the tryptophane emission of the antibody is restricted to those systems which fulfil the spectral conditions for energy transfer by the coupled oscillator mechanism. The widely studied azobenzene systems and the dinitrophenyl system fall within this category. Application to protein antigens is feasible only if the antigen or antibody carries a prosthetic group that can act as acceptor in the energy transfer process. This function was performed by the hapten in the DNP antigen and the solubility of the complex was maintained by using univalent antibody. A natural protein that might be used in the same way as hemoglobin. The heme group quenches nearly all of the fluorescence of the globin and might quench the fluorescence of an antibody in a soluble hemoglobin antibody complex.

The usefulness of the quenching of antibody fluorescence is severely limited in crude systems such as whole serum where the antibody contributes only a small fraction of the total protein emission. In order to take advantage of fluorescence spectroscopy in such cases one might study antibodies directed against fluorescent haptens. The experiments would then be analogous to the enzyme coenzyme systems and one could utilize shifts in the emission spectrum, changes in fluorescent quantum yield, or increases in fluorescence polarization of the hapten as indicators of complex formation. Effects of this type have been described and utilized[1] and are subject to considerable refinement. An obstacle to such an approach would be the occurrence of nonspecific binding to proteins such as serum albumin. Structural properties which confer high fluorescence efficiency often make a molecule susceptible to strong nonspecific adsorption.

In terms of the antigen template hypothesis of antibody formation Pauling[12]

had suggested that complexes of antibodies with "good" antigens should all exhibit dissociation constants within a limited range of values. Too large a dissociation constant would correspond to a weak or nondetectable antibody and too small a dissociation might retard the release of the antibody from its site of origin, and hence limit both the rate of antibody production and the levels of circulating antibody. Although the dissociation constants of the anti-DNP antibody are orders of magnitude smaller than those commonly observed, DNP is a potent determinant, leading to high levels of strong antibody in the plasma. It is unlikely that the proposed simple equilibrium conditions govern the rate of antibody formation or the levels of accumulation in the blood.

Heterogeneity of antibodies is an immunochemical doctrine that is well established in certain cases and assumed to be general. The major question concerning heterogeneity is the extent to which it is the result of induction by a heterogeneous antigen and the extent to which it represents a fundamental randomness in the mechanism of antibody or γ-globulin synthesis. In general, antigens are not completely defined chemically and they may also undergo an unknown number of chemical transformations metabolically before they act. Heterogeneity by heterogeneous induction is chiefly a measure of the organisms ability to respond to small differences in the inducer. If the latter factor were predominant one might expect to find a case in which both the acting inducer and the resulting antibody were uniform and such a case has not yet been found. The relatively high uniformity in the behavior of the purified anti-DNP antibody appears in part to be the result of selection during the purification procedure. The evidence for this, as yet incomplete, is provided by the data of Farah *et al.*[3] in which hapten concentrations necessary to inhibit precipitation or to dissolve antigen antibody precipitates are compared for total antisera and for purified antibody. It is nevertheless significant that purified antibody of the homogeneity level obtained comes in 30 to 40 per cent yield from the pooled antisera of about 20 rabbits. The microheterogeneities responsible for the reversible electrophoretic boundary spreading are possibly more fundamental and it will be necessary in this connection to consider variations in primary as well as in secondary or tertiary structure. Variations in primary structure have been postulated by Lederberg[13] in his elaboration of the elective theory of antibody induction.

Summary.—(1) The tryptophane fluorescence of proteins is quenched in complexes or conjugates of the proteins with molecules that have absorption bands in the 300 to 400 mμ region. This phenomenon, applied previously to the study of enzyme coenzyme complexes is also applicable to antibody complexes with appropriate antigens or haptens. (2) The fluorescence quantum yield of the antibody directed against the 2,4-dinitrophenyl group (DNP) is diminished about 70 per cent by the binding of ϵ-N-DNP-lysine and serves as a sensitive indicator of complex formation. (3) The binding stoichiometry is 2 moles of hapten per mole of purified antibody and the dissociation constant at 7° is 2.4×10^{-9} M. Dissociation constants of this magnitude are not approachable by other methods. (4) Hapten binding has been studied over the pH range 1.5 to 11 and over the temperature range 7 to 60°. (5) The thermodynamic constants, $\Delta H°$ and $\Delta F°$ for complex formation are -11.3 and -8.6 kcal mole^{-1} respectively and the standard entropy change is $\Delta S°$ is 9 eu. (6) Weaker binding of some homologous univalent haptens

and the formation of linear aggregates with a divalent hapten are described. (7) It is established by direct titration that the soluble fragments I and II produced by papain proteolysis of the purified antibody, are univalent antibody pieces and that the crystalline fragment III, prepared by Porter's method, carries no hapten binding site. (8) The univalent antibody fragments may be titrated quantitatively, by the fluorometric method, with intact multivalent DNP antigen since they form soluble complexes in which the fluorescence both of the antibody and antigen is quenched by the DNP ligands on the antigen. (9) The binding sites of the purified anti-DNP antibody exhibit a high degree of homogeneity in their interactions with haptens but the statistical inhomogeneities, in reversible electrophoretic boundary spreading are of the same magnitude as those of nonspecific γ-globulin. (10) The fluorescence quenching method is also applied to the study of hapten interactions of an antibody directed against derivatives of *p*-azophenylarsonate. (11) In the quenching interaction within the hapten complex each molecule of bound DNP captures the excitation energy of 8 to 9 tryptophane residues. These groups of tryptophane residues of the antibody lie within separate domains which do not overlap and the boundaries of which coincide roughly with the points of proteolytic cleavage by papain.

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† Fellow of the Allergy Foundation of America.

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STRUCTURAL ALTERATIONS IN CRYSTALLINE GLUTAMIC DEHYDROGENASE INDUCED BY STEROID HORMONES

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Recently we reported[1] that the glutamic dehydrogenase (GDH) reaction, catalyzed by the crystalline beef liver enzyme or by mitochondrial extracts of rat liver, was inhibited by steroids and diethylstilbestrol (DES), and that this inhibition could be reversed by ADP. Because of the physiological importance of this enzyme, we have extended our investigations in an attempt to determine the mechanism of the inhibition.

In the work to be presented, kinetic experiments suggested a rather complicated interaction between the enzyme and the steroids.[2] In view of the interesting findings of Frieden[3, 4] that DPNH and various inhibitors of GDH could dissociate the protein into enzymically inactive subunits, we were interested in whether the steroid inhibitors could induce similar changes.

Our data show that various steroid hormones can, indeed, produce alterations in the physical state of the enzyme, as reflected in sedimentation characteristics.

Materials and Methods.—Crystalline beef liver GDH, suspended in Na_2SO_4 solution, and the nucleotides were obtained from the Sigma Chemical Company. Steroids and DES were obtained from various commercial sources.

The rate of oxidation of glutamate by either TPN or DPN at room temperature was followed spectrophotometrically by observing the change in optical density at 340 mμ, between 15 and 30 sec, as described by Frieden.[3] The reduction of α-ketoglutarate was likewise observed spectrophotometrically and the initial rate was again calculated from the change between 15 and 30 sec after the start of the reaction, although it followed zero order kinetics for a longer period.

Steroids were dissolved in 50% propylene glycol and the solutions were maintained at 100° before addition to the reaction mixtures in order to prevent precipitation. In the kinetic experiments, the final concentration of propylene glycol in the reaction mixture was 2% by volume, which did not affect the rate in either direction.

Before ultracentrifugation, the crystalline GDH was dissolved in 0.05 *M* tris (hydroxymethyl) aminomethane-HCl buffer, pH 8, containing 5×10^{-4} *M* ethylenediamine tetraacetic acid (EDTA) and the solution was maintained at 0°. Steroids were added in 50 per cent propylene glycol as described above, and appropriate controls were run with propylene glycol alone.

Sedimentation experiments were performed in the Spinco Model E analytical ultracentrifuge,[5] equipped with automatic temperature control, at a rotor speed of 59,780 rpm. Photographs were taken at 4-min intervals, and sedimentation coefficients, calculated from 4 exposures, were correct to 20°, assuming that the viscosity of the solvent (containing propylene glycol) varied with temperature in the same way as that of water. This assumption seemed justified by the observation that $S_{20,w}$ of the enzyme was not changed significantly when the propylene glycol concentration was doubled.

The conditions for each experiment are presented in the legends of the corresponding figure.

Results.—Kinetic experiments: Figure 1 shows the effects of increasing concentrations of steroids on the oxidation of glutamate by TPN. Similar results were obtained when DPN was the oxidizing agent. DES, estradiol, and progesterone produced 50% inhibition at 2×10^{-6}, 3.5×10^{-5}, and 7.2×10^{-5} *M*, respectively. Corticosterone was practically inert as an inhibitor. The relative potency of a more extensive list of steroids was reported in our earlier communication.[1]

When the reciprocals of the reaction rates depicted in Figure 1 were plotted against the corresponding concentrations of estradiol, the curve was progressively deflected upwards at higher inhibitor concentrations (Fig. 2). Data obtained from an examination of the effects of varying concentrations of estradiol on α-keto-

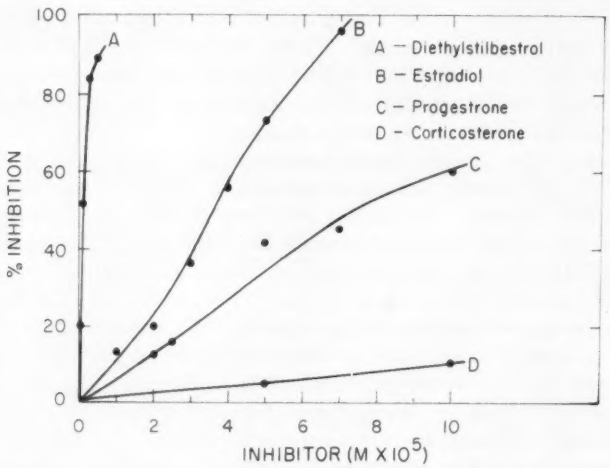

FIG. 1.—The reaction contained tris buffer pH 8 0.01 *M*, 1×10^{-4} *M* EDTA, 2×10^{-4} *M* TPN, 0.025 mg GDH added as a 1:20 dilution of the enzyme suspension in 0.025 *M* tris pH 8 containing 2.5×10^{-4} *M* EDTA, and the appropriate steroid added in propylene glycol as described in the text. Reaction volume was 2.5 ml with a light path of 1 cm.

glutarate reduction were plotted in the same manner, and a similar concave curve was obtained. Figure 2 also shows that ADP prevented the steroid inhibition.

In Figure 3, the reciprocals of the velocities are again plotted against inhibitor concentrations. In this case, the inhibitor was DES and the experiments were conducted with two different amounts of enzyme. It appears that at the higher protein level, the enzyme was less responsive to steroid inhibition than at the lower concentration.

Since the dissociation of the enzyme into inactive subunits is dependent on the protein concentration, the observed variation of the degree of steroid inhibition with protein concentration suggested that the steroids were favoring the reversible disaggregation of the GDH molecule.

Sedimentation experiments: When the sedimentation of crystalline beef liver

GDH was examined in the analytical ultracentrifuge in the presence of 1×10^{-4} *M* DPNH, a single sharp peak was observed (Fig. 4*A*,) with an $S_{20,w}$ of 26.2, which compares with the value, at zero protein concentration, reported by Olson and Anfinsen[6] of 26.6. When 2.5×10^{-4} *M* DES was included in the mixture (Fig. 4*B*, *C*), there was a marked alteration in the sedimentation pattern. Two peaks appeared with $S_{20,w}$ values of 12.5 and 21.7 respectively. The sedimentation coefficient of 12.5 corresponds to that of the small subunit identified by Frieden.[3] It is also interesting that the $S_{20,w}$ value of 21.7 is less than that of the single component observed in the absence of steroid. These two components were also seen when the DES concentration was reduced to 5×10^{-5} *M*, although the dissociation was less at the lower concentration.

Estradiol and progesterone, both inhibitors of the GDH reaction, were examined

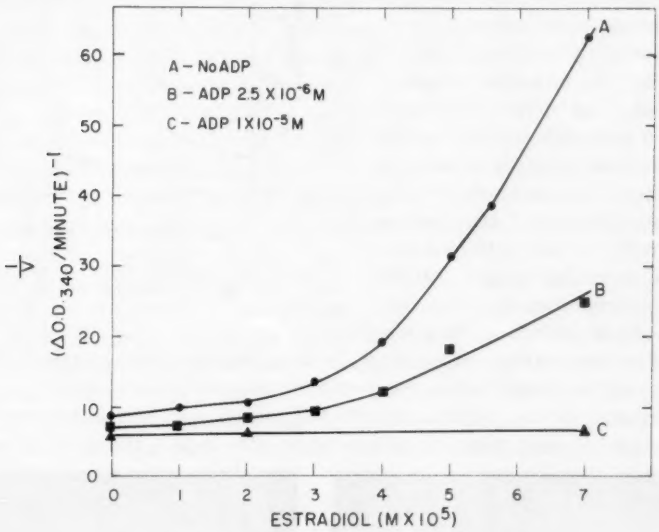

FIG. 2.—Experiment conducted as in Figure 1.

for their effects on the behavior of GDH in the ultracentrifuge. At 2.5×10^{-4} *M*, they produced changes which were qualitatively similar to those seen with DES. When the areas of the slower peaks were compared, it was apparent that DES was the most, and progesterone the least, effective in splitting the enzyme. These findings are correlated with the relative inhibitory effects of these compounds on the GDH reaction. In addition, cortisone, which does not inhibit the reaction, did not produce any visible splitting of the enzyme at 2.5×10^{-4} *M*.

Since Frieden[3] has reported that DPNH, in concentrations above 4×10^{-4} *M*, can cause the enzyme to dissociate, it was important to find out whether the steroids were merely enhancing the effect of the reduced pyridine nucleotide. Even when DPN was substituted for DPNH, 2.5×10^{-4} *M* DES produced 2 peaks with sedimentation coefficients of 13.3 and 21.0. Thus DPNH was not required for the steroid-induced splitting of the enzyme. When 2.5×10^{-4} *M* DES was tested

alone, although only one peak was observed, its trailing edge was broader that in the control cell. Both boundaries had approximately the same sedimentation coefficient, i.e., 21.9 for the sample run with DES and 20.8 for the control.

Frieden[4] also showed that ADP protected GDH against dissociation by DPNH or 1,10-phenanthroline. Since our studies had shown that ADP could reverse the steroid inhibition of the GDH reaction,[1] we examined the effect of 5×10^{-4} *M* ADP on the dissociation of the enzyme induced by 2.5×10^{-4} *M* DES (in the presence of 1×10^{-4} *M* DPNH). Figure 4*D* shows that ADP virtually prevented the dissociation.

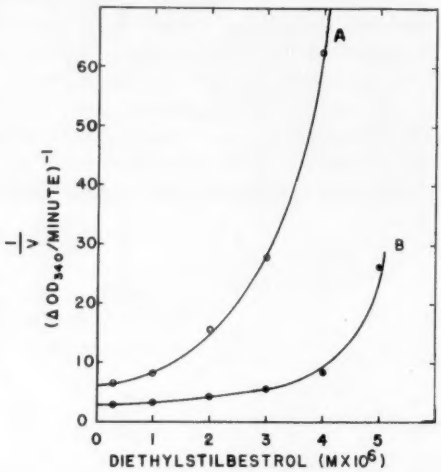

FIG. 3.—Experiment conducted as described for Fig. 1. *A:* GDH, 0.004 mg/ml. *B:* GDH, 0.012 mg/ml.

Discussion.—Olson and Anfinsen[6] found that the molecular weight of crystalline beef liver GDH was 1,000,000 and that, at low protein concentrations, it either dissociated or unfolded. Subsequently, Frieden presented evidence[3, 4] that the enzyme could be split into four subunits, of molecular weight 250,000 by various inhibitors and high concentrations of DPNH. There was an excellent correlation between the state of aggregation of the protein and its catalytic activity, which led him to conclude that only the intact molecule was active.

The kinetics of the inhibition of the GDH reaction by steroid presented in this paper suggested that these hormones might also induce the dissociation of the

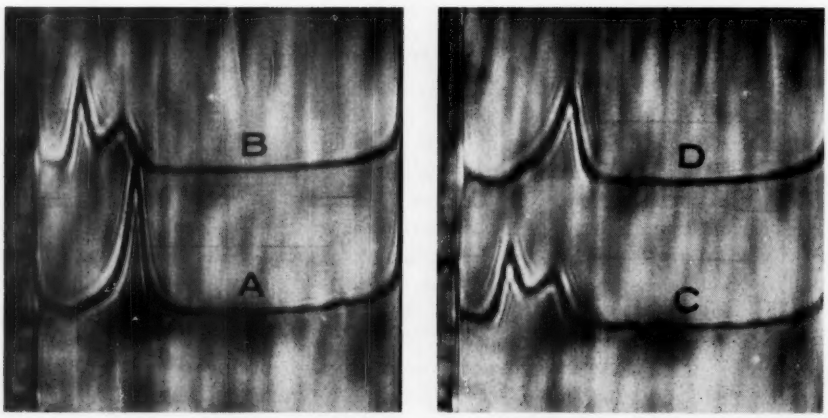

FIG. 4.—*A:* control. *B, C:* DES, 2.5×10^{-4} *M*. *D:* DES, 2.5×10^{-4} *M* and ADP, 5×10^{-4} *M*.

Each reaction mixture contained GDH 5 mg/ml, 2.5% propylene glycol, and 1×10^{-4} *M* DPNH. All experiments were conducted between 5 and 12°.

enzyme into subunits and the sedimentation velocity experiments, also presented, confirmed this impression. There is, furthermore, a correlation between the ability of the steroids tested to inhibit the reaction and their capacity to promote the disaggregation. In addition, cortisone produced neither splitting nor inhibition of the enzyme. ADP prevented both steroid inhibition and steroid-induced dissociation, which was also consistent with the idea that the change in the physical state of the molecule was responsible for the inhibition.

The intimate mechanism by which the steroids affect either the sedimentation or the kinetics of the enzyme is unknown, although it is clear from the data that they do not merely enhance its DPNH-induced dissociation. Munck[7] has called attention to the orientation of steroids at polar-nonpolar interfaces, and it is conceivable that surface forces such as he considered may explain our results.[8]

There are, in general, two classes of ideas about the ways in which steroid hormones catalytically affect enzymic reactions. In the first, the steroid itself is thought to be chemically altered during the reaction. This is exemplified by Talalay and Williams-Ashman's concept of the role of steroids in the transfer of hydrogen between DPN and TPN.[9] They hypothesized that there is alternate oxidation and reduction of the oxygen functions on the steroid nucleus to account for the transhydrogenation reaction. Another interesting example of this class of steroid effects was proposed by Roy[10] who observed steroid-stimulation of arylamine sulfate synthesis from "active sulfate," and suggested that enol-sulfates of steroid ketones were intermediates in sulfate transfer.

In other instances, as with the present case, the steroids appear to affect the activity of enzymes without themselves being chemically changed. Examples of this are the alterations in the activity of the steroid-sensitive aldehyde dehydrogenase,[11] inhibition of glucose-6-phosphate dehydrogenase,[12] inhibition of DPNH-cytochrome c reductase,[13–15] and activation of latent mitochondrial ATP'ase,[16] and inhibition of 3-phosphoglyceraldehyde dehydrogenase by ergosterol.[17] In the last named example, the authors suggested that a physical change in the enzyme was responsible.

The present findings that steroids can alter the state of aggregation of a protein suggest a general mechanism by which these hormones might affect the catalytic behavior of other enzymes.

Summary.—1. Diethylstilbestrol, estradiol, and progesterone, all of which inhibit the glutamic dehydrogenase reaction, promoted dissociation of the crystalline beef liver enzyme into subunits as determined by ultracentrifugation.

2. Cortisone, inactive as an inhibitor, did not influence the behavior of the protein in the ultracentrifuge.

3. ADP could prevent both the steroid inhibition and steroid–induced disaggregation of the enzyme.

4. It is, therefore, suggested that the change in the structure of the enzyme induced by steroids is responsible for the action of the hormones as inhibitors of crystalline beef liver glutamic dehydrogenase.

We should like to acknowledge the excellent assistance of Mrs. Janet S. Munday in performing many of the experiments in this communication.

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POTASSIUM GLUCONATE AND ATP EFFECTS ON CHROMOSOME ABERRATION YIELD

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Beatty and Beatty[1, 2] suggested that in *Tradescantia* the initial damage from a given dose of X-radiation in any experimental design probably is the same, and that the various yields of chromosome aberrations are more dependent upon the recovery mechanism involved in chromosome rejoining than on the breakage phenomenon. If 400 r of X-radiation is given to developing microspores and chromosome rejoining is inhibited, a high yield—presumably a maximum—of recoverable two-hit aberrations of the ring and dicentric type is obtained. For example, irradiation in 5 per cent oxygen in CO at any intensity from 1 r to 400 r per min and/or at any temperature between 0° and 45°C, will give aberration yields around 0.88 per cell. In helium, irradiation at 40°C (at 50 r or 400 r per min), at 30°C (at 1 r per min), or irradiation at 50 r per min following 400 min pretreatment in anoxia, will give aberration yields around 0.66 per cell. On the other hand, if the experimental procedures include factors favorable for rejoining, the aberration yields are much lower. For example, a total dose of 400 r given in helium at 0°C, will give an aberration yield of 0.14 per cell in comparison to 0.66 at 40°C. Post-irradiative application of the influential factors has demonstrated that in *Tradescantia*, at least, they operate on the recovery mechanism and are associated with the supply of energy necessary for recovery from radiation damage.

Oxidative metabolism appears to be responsible for the supply of energy for chromosome repair in an aerobic system and can be inhibited by CO.[2] CO has no effect, other than producing anoxia, in an anaerobic system, so the energy must

come from some source not responsive to the action of CO.[1] Beatty and Beatty[1] suggested that in an anaerobic system the glycolytic pathway, perhaps, furnishes the energy for chromosome rejoining. This hypothesis was tested by using anaerobic respiratory inhibitors on microspores irradiated in helium. Finding that a high of 0.66 aberrations per cell was obtainable with 400 r given in helium, while the controls (at 30°C) gave 0.24 per cell and that the inhibitors did not increase the yield above 0.55 aberrations per cell, they concluded that, although the glycolytic system seemed to be the primary pathway involved in chromosome repair in an anaerobic system, some other energy pathway must be involved. The hexose monophosphate pathway (HMP) seemed to offer the best possibility. Many plants and animals have capabilities of utilizing this system of energy transformation. Gibbs[3] in a review of metabolism of carbon compounds lists several such organisms: pea, corn, and oat seedlings; wheat and barley leaves; *Leuconostoc* and lactic acid bacterium; nonnucleated erythrocytes; liver, etc. Since ATP is produced through both the glycolytic route and the HMP, its role in modification of aberration production was also studied.

Materials and Methods.—Inflorescences of *Tradescantia paludosa* were removed just prior to use from plants growing in the greenhouse and after treatment returned to the greenhouse in beakers of water, where they remained until acetocarmine preparations of the microspores were made 96 hr later. Chromosome aberrations of the ring and dicentric types were scored.

The experimental temperatures used were 0.2°C, 30°C, and 40°C. In the two temperature extremes, the material was allowed a total of 40 min for adaptation —30 in air and 10 in helium. A total dose of 400 r given at 50 r per min was used in all experiments, which were carried out in the dark.

Freshly prepared solutions of 2×10^{-3} *M* ATP (Schwarz), 1×10^{-2} *M* potassium gluconate (Matheson Coleman and Bell), and 2×10^{-3} *M* sodium 6-phosphogluconate (Sigma) were used. The peduncles of the inflorescences were cut $2^1/_2$ inches from the flower buds and immersed $1^1/_2$ inches in the solution. Pretreatment in the solutions was carried out in air, while in the posttreatment experiments the solutions were contained in a helium-closed system so that they could be emptied

TABLE 1

CHROMOSOME ABERRATION YIELD FOLLOWING 400 R OF X-RADIATION AT 50 R PER MINUTE WITH TREATMENT IN ATP, POTASSIUM GLUCONATE, OR 6-PHOSPHOGLUCONATE

Experiment no.	Temp.	Treatment		No. cells	Av. interchanges per cell
1	30°C	Control	...	2,500	0.24–0.009
2	"	ATP	Pre-	500	0.14–0.017
3	"	ATP	Post-	600	0.13–0.015
4	"	Gluconate	Pre-	500	0.13–0.016
5	"	Gluconate	Post-	500	0.15–0.017
6	"	6-Phosphogluconate	Pre-	500	0.12–0.016
7	"	ATP-Gluconate	Pre-	600	0.10–0.013
8	40°C	Control	...	1,000	0.66–0.026
9	"	ATP	Pre-	500	0.19–0.019
10	"	Gluconate	Pre-	500	0.21–0.020
11	"	6-Phosphogluconate	Pre-	550	0.23–0.020
12	"	ATP-Gluconate	Pre-	500	0.17–0.018
13	0.2°C	Control	...	1,000	0.14–0.012
14	"	ATP	Pre-	700	0.10–0.012
15	"	Gluconate	Pre-	500	0.11–0.015
16	"	6-Phosphogluconate	Pre-	500	0.09–0.014
17	"	ATP-gluconate	Pre-	500	0.10–0.014

into the beaker containing the cut peduncles within 7 sec after irradiation. Both pretreatment and posttreatment periods were $1^1/_2$ hr duration.

Results and Discussion.—The results of previous experiments have shown that 400 r of X-radiation given at 50 r per min to *Tradescantia* microspores in a helium atmosphere gave aberration yields which varied with the temperature.[1] Three control experiments were therefore carried out (Table 1) at temperatures of 30°C, 40°C, and 0.2°C, resulting in aberration yields of 0.24 (No. 1), 0.66 (No. 8), and 0.14 (No. 13), respectively. This temperature effect is believed to be due to an energy balance within the cell, so that less energy is used in basic cell metabolism at low temperatures, leaving more energy available for chromosome rejoining, while the reverse would be true for high temperatures. These three experiments, besides being controls for each temperature area investigated, form base lines useful in comparing the aberration yields of treated microspores.

In experiment 2 (at 30°C) where pretreatment was in ATP, an aberration yield of 0.14 per cell was recorded in comparison to 0.24 (No. 1) of the control. The nature of this effect is further revealed in experiment 3, where a similar reduction in aberration yield was found, 0.13 per cell, but the ATP treatment was postirradiative. The "protective" nature of ATP thus appears to be not on the chromosome breakage aspect of radiation damage, but on the recovery or repair mechanism.

In control experiment 8 the material was irradiated at 40°C and gave an aberration yield of 0.66 per cell, while in experiment 9 at the same temperature microspores pretreated in ATP yielded an aberration count of 0.19 per cell—giving a greater percentage reduction in aberrations than was recorded at 30°C. At 0.2°C the control experiment (No. 13), gave an aberration yield of 0.14, while material pretreated with ATP gave a yield of 0.10 aberrations per cell (No. 14). An ATP effect was thus evident at each of the three temperatures used.

A number of experiments not shown in Table 1 were carried out at 30°C using different periods of pretreatment with ATP. The greatest effect began at the 1 hr period and remained the same, 0.14 aberrations per cell, through 9 hr of pretreatment. Also, doubling the concentration for $1^1/_2$ hr pretreatment at 30°C did not increase the effectiveness of ATP.

Radioactive ATP was used to test further the passage of the molecule or part of the molecule into the microspore material. In the ATP used, carbon number 8 was radioactive. Because of the low activity of the solution used, the results were not startling but still suggestive. The total number of counts used in assaying was 320 per min. Five lambda of the stock solution gave this yield in 0.04 min. The background count was around 13 min for this total count. Six anthers from flower buds containing cells in the first microspore division were smeared on the disk. All extraneous material was removed, leaving only the developing microspores. Six such samples were prepared at each time interval from material pretreated up to 4 hr in radioactive ATP. The shortest time required to obtain the necessary count was 3 min and this occurred in some material which had been pretreated from 2 to 4 hr. In the control experiment, designed to test for biological activity, the material was pretreated for $1^1/_2$ hr before irradiation and gave an aberration yield of 0.14 per cell. Biological activity thus was registered before radioactivity was evident in the microspore.

The potassium salt of gluconic acid was used to investigate other possible energy

pathways in addition to the glycolytic system, which might operate in *Tradescantia* to furnish energy for repair from radiation damage. In the first such experiment (No. 4) carried out at 30°C, influorescences pretreated in potassium gluconate were found to yield aberrations averaging 0.13 per cell in comparison to 0.24 of the control (No. 1). Similarly, a postirradiative treatment lowered the aberration yield to 0.15 per cell (No. 5). Irradiation at 40°C of pretreated material (No. 10) resulted in a lowering of aberration yield from 0.66 per cell of the control (No. 8) to 0.21. A similar pretreatment of material irradiated at 0.2°C reduced the aberration yield to 0.11 per cell (No. 15) in comparison to 0.14 of the control (No. 13). As with ATP the effectiveness of potassium gluconate is apparently on the recovery mechanism rather than on the breakage phenomenon.

Another compound, 6-phosphogluconate, which is involved in one or more of the alternate pathways of glucose metabolism, was used in three experiments. Pretreated material irradiated at 30°C gave an aberration yield of 0.12 per cell (No. 6) in contrast to 0.24 of the control (No. 1). Irradiation at 40°C of pretreated material lowered the average aberrations from 0.66 per cell (No. 8) of the control to 0.23 for this experiment (No. 11). In experiment 16, pretreated microspores irradiated at 0.2°C, the aberration yield was reduced from 0.14 per cell for the control (No. 13) to 0.09 for the treated. This compound thus reduced the aberration yield at all three temperatures about the same degree as found in material treated with ATP or gluconate.

Experiments 7, 12, and 17 were carried out to determine if ATP and potassium gluconate used simultaneously might have some synergistic effect within the cell, so that an even lower aberration yield might be obtained than was found after individual use of the same compounds. In experiment 7 pretreatment and irradiation were at 30°C. In control experiments (No. 1) the yield was 0.24 per cell while the treated microspores yielded an average of 0.10 chromosome aberrations per cell, which is lower than was obtained in either experiments 2 or 4. With irradiation at 40°C, the aberration yield was reduced from 0.66 for the control (No. 8) to 0.17 per cell (No. 12). The individual treatments gave 0.19 (No. 9) for ATP and 0.21 for gluconate (No. 10). At 0.2°C, with the control yielding 0.14 (No. 13), the experimental result was 0.10 per cell (No. 17). The individual treatments resulted in 0.10 (No. 14) for ATP and 0.11 (No. 15) for gluconate at this temperature. The synergistic effect appears to decrease with lowered temperature since at 0.2°C no synergism was observed.

Although an additional supply of exogenous ATP or an endogenous modification through the glycolytic or hexose monophosphate pathway have been shown to have an effect on chromosome aberration yield in *Tradescantia*, the results should not be interpreted as positive proof that ATP is directly involved in the repair of chromosome damage induced by irradiation. It would seem more appropriate at the present time to consider that there is an energy pool within the cell from which energy is used in many ways and in which ATP plays an important role. In these experiments the ATP was released into the pool from more than one pathway. Since chromosome repair is a requirement above the normal metabolic processes within the cell, any additional flow of energy into the pool would make more energy available for this imposed extracurricular activity of recovery from radiation damage.

In some current work using the same experimental procedures employed in these investigations, glucuronic acid, ribose 5-phosphate, or sodium pyruvate had very little influence on aberration yield. This, in addition to the fact that in all these experiments the Krebs cycle was blocked by anoxia, strongly suggest that the HMP in some way is or can be involved in repair from radiation damage. This pathway would produce several times the amount of ATP provided by glycolysis.

The posttreatment experiments with gluconate and ATP clearly demonstrate that the "protective" nature of these compounds is due to their effect on the recovery mechanism rather than on the breakage phenomenon. These results suggest that, in most cases, an approach to minimizing radiation damage through the recovery mechanism would be more practical and profitable than attempting to modify the initial radiation damage. They also indicate that the activities and physiology of the cell are important variables and could easily modify the results in studies dealing with the biological effects of radiation.

Summary.—Aberration yields from *Tradescantia* microspores subjected to 400 r of X-radiation and pre- or posttreated in chemical compounds were determined. In material irradiated at 30°C, the control yielded 0.24 aberrations per cell while material treated in ATP gave 0.14, in potassium gluconate 0.13, and in 6-phosphogluconate 0.12. Material simultaneously treated in ATP and gluconate gave 0.10. Material irradiated at 40°C gave 0.66 for the controls, 0.19 for ATP, 0.21 for gluconate, 0.23 for 6-phosphogluconate, and 0.17 for the simultaneous treatment with ATP and gluconate. Material irradiated at 0.2°C gave 0.14 for the control, 0.10 for ATP, 0.11 for gluconate, 0.09 for 6-phosphogluconate, and 0.10 for the simultaneous use of ATP and gluconate. The postirradiative treatment with ATP or gluconate indicated that these compounds act on the recovery mechanism of radiation damage. Both the glycolytic and the hexose monophosphate pathways appear capable of releasing energy for repair from radiation damage.

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*GENETIC CONTROL OF THE α- AND β-CHAINS OF HEMOGLOBIN**

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Identical pairs of two chemically different polypeptide chains, the α-chains and the β-chains,[1, 2] make up the protein portion of human adult hemoglobin. Hemoglobin A, in which both types of chains are normal, is designated $\alpha_2^A\beta_2^A$. Either type may carry the inherited defect of an abnormal hemoglobin; the specific amino acid substitution of hemoglobin S or C occurs in the β-chain, and the substitution of hemoglobin I occurs in the α-chain.[3, 4] These abnormal adult hemoglobins are

designated $\alpha_2^A\beta_2^S$, $\alpha_2^A\beta_2^C$, and $\alpha_2^I\beta_2^A$, respectively. The biosynthetic and structural bases for stable pairing of identical chains is as yet unexplained. Free exchange of chain-pairs occurs when a mixture of hemoglobins is dissociated and recombined to produce, for example, $\alpha_2^A\beta_2^A$ and $\alpha_2^I\beta_2^S$ from $\alpha_2^A\beta_2^S$ and $\alpha_2^I\beta_2^A$;[5] on the other hand hybrid molecules of the type $\alpha^A\alpha^I\beta_2^A$ or $\alpha_2^A\beta^A\beta^S$ have not been detected in hemolysates or in recombined mixtures.

Allelic genes control the synthesis of hemoglobins A, S, and C,[6] which differ from each other only in their β-chains,[3, 4] homozygosity at this locus resulting in the presence of one adult hemoglobin, and heterozygosity, in two. The absence of hemoglobin A in sickle cell-hemoglobin C disease, in fact, furnished the initial clue to allelic control.[7] Whether or not other loci participate in the genetic control of hemoglobin synthesis has been the subject of much discussion. Recent speculation has centered about the more specific question of whether or not different loci control the α-chains on the one hand and the β-chains on the other; however, critical data bearing on this point have heretofore been unavailable.

The present study stems from the discovery by Smith and Torbert[8] that hemoglobin Hopkins-2 (Ho-2) is determined at a locus different from the one which determines hemoglobin S. The simultaneous presence of sickle cell trait and hemoglobin Ho-2 trait in the same individual (an abnormal gene and its normal allele at each locus) is associated with the presence of three hemoglobin components, the two abnormal hemoglobins and a component with the electrophoretic mobility of hemoglobin A. The findings of Smith and Torbert raise questions pertinent to the mode of genetic control of synthesis of the chains of hemoglobin. Are hemoglobins Ho-2 and S defective in different chains? If so, why does double heterozygosity result in only three electrophoretic components? Formation of four molecular species, normal and doubly abnormal as well as two singly abnormal, would be expected to result from independent synthesis of a normal and an abnormal α-chain and a normal and an abnormal β-chain in the same red cell. The present paper reports studies of these problems by the technique of asymmetric recombination of of hemoglobin.[9] Further familial studies on the kindred reported by Smith and Torbert[8] have been conducted by Bradley, Boyer, and Allen.[10]

Materials and Methods.—Hemoglobin solutions: Carbonmoxyhemoglobin (HbCO) was prepared from the blood of members of the kindred with hemoglobins S and Ho-2. Washed red cells were lysed by dialysis at 5° in a CO atmosphere against several changes of water. The hemolysate was electrodialyzed, clarified by centrifugation, and saturated with CO. Previously prepared stock solutions of HbCO S and C and of a mixture of HbCO A and I were also used in the present studies.

Chromatographic separations: Fractions for recombination experiments were prepared by chromatography on 5 × 35 cm and 5 × 45 cm columns of the cation exchange resin, Amberlite CG-50, Type 2,[11] at 5°. The columns were prepared and the HbCO samples applied as described by Allen, Schroeder, and Balog.[12] 1–2 g of HbCO in 20–30 ml of water was chromatographed in each experiment. The stock solution of developer was a sodium phosphate buffer at 0.5 M Na^+ concentration, which contained 27.6 g $NaH_2PO_4 \cdot H_2O$, 21.3 g Na_2HPO_4, and 0.65 g KCN per liter. The developer was diluted to 0.1 M Na^+, at which concentration its pH is 6.7, for initial equilibration of the columns. The samples were eluted at this

concentration until the first steady minimum of HbCO in the effluent was reached. The second main hemoglobin zone was eluted with stock developer diluted to 0.15 *M* Na^+, and the last zone was eluted with developer diluted to 0.20 *M* Na^+. The flow rate was 20–40 ml/hr, and the volume of each fraction was 20 ml. Concentrations of the fractions were determined from absorbancy readings at 538 mμ. Fractions corresponding to the major zones of elution were pooled and dialyzed against water or dilute neutral phosphate buffer and then concentrated by pervaporation. The concentrates were treated with $Na_2S_2O_4$ and CO to convert the ferrihemoglobin produced during pervaporation to HbCO, dialyzed against water, and clarified by centrifugation.

Recombination experiments: Samples were analyzed before and after recombination by moving boundary electrophoresis at 0.5° in potassium phosphate buffer of pH 6.8, μ 0.02 for 5 hr at a field strength of 9.3 v/cm. Recombination of HbCO was carried out by dissociation of mixtures at 1 g/100 ml concentration at pH 4.7 or 11.0 for either 4 or 24 hr followed by dialysis against three changes of the phosphate buffer used for electrophoretic analysis.[9, 13]

Results and Discussion.—Samples of HbCO from hemoglobin Ho-2 trait (HbCO A and Ho-2), double heterozygosity for the genes that control hemoglobins S and Ho-2 (HbCO S, Ho-2, and A-like component), and the mixture of HbCO A and I used in an earlier study[5] were analyzed by column chromatography. HbCO Ho-2 and I were eluted with developer at 0.1 *M* Na^+, HbCO A and A-like component of double heterozygotes at 0.15 *M* Na^+, and HbCO S at 0.20 *M* Na^+. Although completely homogeneous preparations were not obtained,[14] the proportion of

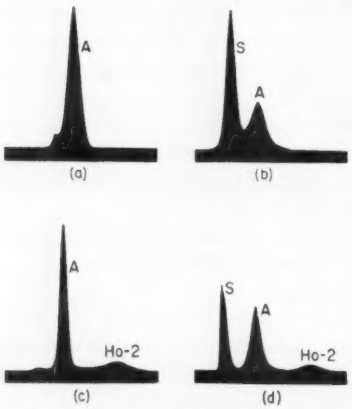

FIG. 1.—Ascending limb electrophoretic patterns of 1% HbCO solutions of the four genotypes of the Fuller kindred in pH 6.8, μ 0.02 phosphate buffer. The direction of migration is from right to left. (*a*) Normal, (*b*) Strait, (*c*) Ho-2 trait (*d*) Ho-2-S double heterozygote.

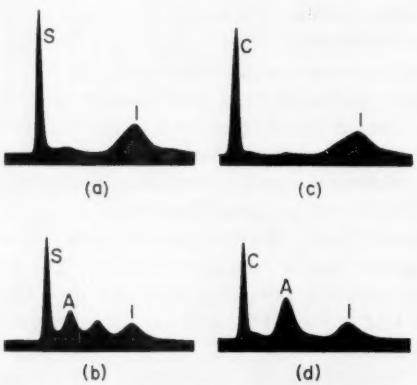

FIG. 2.—Ascending limb electrophoretic patterns of 1% HbCO in pH 6.3, μ 0.02 phosphate buffer. The direction of migration is from right to left. (*a*) I + S control (*b*) I + S recombined (*c*) I + C control (*d*) I + C recombined.

desired component was in each case sufficiently high to permit satisfactory recombination experiments.

Samples from each of the four genotypes in this kindred were analyzed by moving boundary electrophoresis (Fig. 1). The discovery of Smith and Torbert, that

the hemoglobin of the S-Ho-2 double heterozygote contains an intermediate component with the mobility of hemoglobin A, was confirmed. In the buffer of pH 8.6 used by Smith and Torbert, in which the hemoglobins are negatively charged, Ho-2 migrates more rapidly than A, and A migrates more rapidly than S. At pH 6.8 each of the hemoglobins carries a positive charge. HbCO C has the greatest electrophoretic mobility, and HbCO S, A, Ho-2, and I follow in order.

When HbCO S and I were recombined, two components, one with the mobility of A and the other with mobility between those of A and I, were produced; when HbCO C and I were recombined, one component with the mobility of A was produced (Fig. 2). The amount of the single product of the C-I recombination was, however, as great as the sum of the amounts of the two products of the S-I recombination (Table 1). Recombination of HbCO S and Ho-2 resulted in net formation of one intermediate electrophoretic component, and recombination of HbCO C and Ho-2 resulted in net formation of two intermediate components which were poorly resolved (Fig. 3). One of the two products of the C-Ho-2 recombination has the mobility of HbCO A, and the other has a slightly higher mobility. The total increase of the single product of the S-Ho-2 recombination was approximately equal to that of the two products of the C-Ho-2 recombination taken together (Table 2). Net formation of a component with the mobility of HbCO S always resulted from recombination of the intermediate component of the S-Ho-2 double heterozygote (Fig. 4 and Table 3). A component with the approximate mobility of HbCO Ho-2 remained as a contaminant of the intermediate component after chromatographic separation. This component either increased or decreased in different experiments; however, net change in amount was never large. No significant change was observed on recombination of the A-Ho-2 mixture of

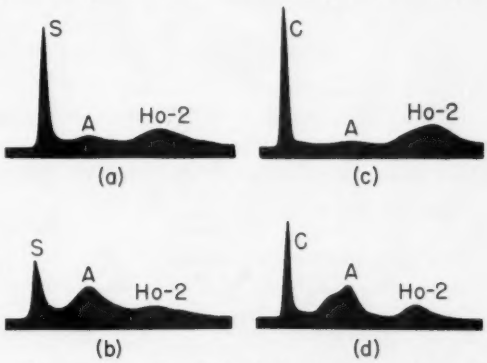

FIG. 3.—Ascending limb electrophoretic patterns of 1% HbCO in pH 6.8, μ 0.02 phosphate buffer. The direction of migration is from right to left. (*a*) Ho-2 + S control (*b*) Ho-2 + S recombined (*c*) Ho-2 + C control (*d*) Ho-2 + C recombined. Note component migrating ahead of A.

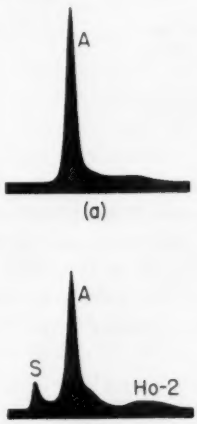

FIG. 4. — Ascending limb electrophoretic patterns of 1% HbCO solutions of the chromatographically isolated intermediate component of the Ho-2-S double heterozygote in pH 6.8, μ 0.02 phosphate buffer. The direction of migration is from right to left.
(*a*) Control.
(*b*) Recombined.

TABLE 1

RECOMBINATION OF HbCO I WITH HbCO S AND C

Mixture		Electrophoretic composition: Per cent of total I	$\alpha_2^I\beta_2^S$	A	S	C
I[1] + S	Control	46	..	6[2]	48	..
	Recombined	23	19	22[2]	36	..
I[1] + C	Control	47	..	3	..	50
	Recombined	22	..	42[3]	..	36

[1] Hb-I was prepared from an A-I sample by column chromatography resulting in a mixture of 94% I plus 6% A. 1 part of this mixture was used to 1 part S or C.
[2] This includes the trailing shoulder of S.
[3] This presumably includes $\alpha_2^I\beta_2^C$.

TABLE 2

RECOMBINATION OF HbCO Ho-2 WITH HbCO A, S, AND C

Mixture	Dissociation[1] time	Electrophoretic composition: Per cent of total Ho-2	A	S	C
Ho-2 trait	Control	21	79	..	..
	24 hr	19	81	..	..
Ho-2 + A + S[2]	Control	42	17	41	..
	4 hr	33	32	35	..
	23 hr	26	47	27	..
Ho-2 + A + C[3]	Control	46	9	..	45
	24 hr	20	47[4]	..	33

[1] All dissociations were done at pH 4.7.
[2] The sample was a 50–50 mixture of S and Ho-2 + A. The latter was prepared from Ho-2 trait by column chromatography, resulting in a mixture of 75% Ho-2 plus 25% A.
[3] The sample was a 55–45 mixture of C and Ho-2 + A. The latter was prepared as above (2), giving a mixture of 90% Ho-2 plus 10% A.
[4] This includes approximately 16% of a faster component, presumably $\alpha^{Ho\text{-}2}\beta_2^C$.

TABLE 3

RECOMBINATION OF INTERMEDIATE COMPONENT OF HO-2-S DOUBLE HETEROZYGOTE

Dissociation pH	Dissociation time	Electrophoretic composition: Per cent of total Ho-2	A	S
Control	..	15	84	1
4.7	4 hr.	17	73	10
Control	..	13	87	..
4.7	4 hr.	12	77	11
4.7	24 hr.	9	79	12
Control	..	14	86	..
11.0	4 hr.	18	73	9
11.0	24 hr.	14	76	10

hemoglobin Ho-2 trait.

The results on the recombination of nearly homogeneous HbCO I with S and C confirm our earlier conclusions based on experiments conducted in the presence of HbCO A.[5] The observed net changes as shown in Figure 2 and Table 1 are consistent with asymmetric recombination of a hemoglobin abnormal in the α-chain with a hemoglobin abnormal in the β-chain to form hemoglobin A (normal adult hemoglobin) and a hemoglobin abnormal in both chains. The net reactions may be written

$$\alpha_2^A\beta_2^S + \alpha_2^I\beta_2^A = \alpha_2^A\beta_2^A + \alpha_2^I\beta_2^S$$

and

$$\alpha_2^A\beta_2^C + \alpha_2^I\beta_2^A = \alpha_2^A\beta_2^A + \alpha_2^I\beta_2^C.$$

Our inference from electrophoretic patterns that the abnormalities in mobilities of HbCO C and I are equal and opposite and that $\alpha_2^A\beta_2^A$ and $\alpha_2^I\beta_2^C$ therefore have

the same mobility is consistent with recent results of Murayama,[15] which have shown that the inherited defect of hemoglobin I is the presence of an aspartic acid residue in a position of the α-chain normally occupied by a lysine residue. With respect to alteration of net charge this substitution is equal and opposite to the substitution in hemoglobin C of a lysine residue for a glutamic acid residue in the β-chain. The negative defect in net charge of hemoglobin I is greater than the positive defect of hemoglobin S; consequently $\alpha_2^{I}\beta_2^{S}$ migrates between HbCO A and I.

The above experiments with hemoglobins known to be abnormal in the α-chain and the β-chain facilitate interpretation of results of experiments with hemoglobin Ho-2. Indeed the suggestion that the intermediate component of S-Ho-2 double heterozygotes might be a mixture of two electrophoretically equivalent species was based on the C-I recombination pattern.[16] Asymmetric recombination of a HbCO mixture does not result in change in electrophoretic pattern when the components of the mixture have a common chain, as do HbCO A, S, and C.[17] The results of Figure 3 and Table 2 thus indicate that hemoglobin Ho-2 differs from S and C in both chains and must be abnormal in the α-chain, the normal chain of S and C. The β-chain of hemoglobin Ho-2 must then be normal, i.e., the same as that of hemoglobin A, since recombination of Ho-2 with A did not produce any new species. Further support for the latter conclusion was furnished by the appearance of a component with mobility of HbCO A after recombination of HbCO Ho-2 with S and C. Since S and C furnished the normal α-chain, Ho-2 must have furnished the normal β-chain. We therefore represent hemoglobin Ho-2 as $\alpha_2^{Ho\text{-}2}\beta_2^{A}$ and write its recombinations with S and C, respectively, as

$$\alpha_2^{A}\beta_2^{S} + \alpha_2^{Ho\text{-}2}\beta_2^{A} = \alpha_2^{A}\beta_2^{A} + \alpha_2^{Ho\text{-}2}\beta_2^{S}$$

and

$$\alpha_2^{A}\beta_2^{C} + \alpha_2^{Ho\text{-}2}\beta_2^{A} = \alpha_2^{A}\beta_2^{A} + \alpha_2^{Ho\text{-}2}\beta_2^{C}.$$

Net formation of only one electrophoretic component when HbCO S and Ho-2 were recombined is consistent with the approximately equal and opposite abnormalities in the mobilities of these hemoglobins (Figs. 1 and 3) and lends support to the hypothesis that the intermediate component of S-Ho-2 double heterozygotes is a mixture of $\alpha_2^{A}\beta_2^{A}$ and $\alpha_2^{Ho\text{-}2}\beta_2^{S}$. The positive abnormality in mobility of HbCO C is greater than the negative abnormality of Ho-2; therefore $\alpha_2^{Ho\text{-}2}\beta_2^{C}$ migrates between A and C.[18]

Presence of a component with the mobility of HbCO Ho-2 in the chromatographically prepared material complicated the results of recombination experiments with the intermediate component of the S-Ho-2 double heterozygote. The same component appeared when a solution of HbCO A was merely diluted and reconcentrated. This component, which migrates more slowly than A at acid pH, corresponds to rapidly migrating material at pH 8.6, which at least in part is derived from hemoglobin A.[19] While the results of Table 3 are conflicting with respect to formation of HbCO Ho-2, they show net formation of material with the mobility of HbCO S in every experiment. Such a component did not appear after recombination of unaltered HbCO A, a mixture of HbCO A and Ho-2, or diluted and reconcentrated HbCO A. The most likely source of the chains that cause

its high mobility is the postulated doubly abnormal molecule, $\alpha_2^{\text{Ho-2}}\beta_2^{\text{S}}$, which has the mobility of HbCO A and can recombine with the latter to produce HbCO S and Ho-2. The postulated net reaction is the reverse of the recombination of HbCO S and Ho-2. The small increases and decreases in proportion of HbCO Ho-2 or a Ho-2-like component observed under different conditions of recombination of the intermediate component probably represent the net effect of two competing reactions. Material was lost on account of irreversible denaturation of the Ho-2-like component derived from HbCO A, and HbCO Ho-2 was produced from the recombination of HbCO A and $\alpha_2^{\text{Ho-2}}\beta_2^{\text{S}}$.

The erythrocytes of sickle cell trait contain more hemoglobin A than S;[7] accordingly the net synthesis of β^{A}-chains must be greater than that of β^{S}-chains in heterozygous cells. Likewise the fraction of hemoglobin Ho-2 in Ho-2 trait of 15–20 per cent[8] must signify that the net synthesis of α^{A}-chains is greater than that of $\alpha^{\text{Ho-2}}$-chains. It follows that the S-Ho-2 double heterozygote would have more normal hemoglobin than doubly abnormal, an inference supported by the results of recombination of the intermediate component, in which the maximum proportion of hemoglobin S produced was 12 per cent.

The familial data of Smith and Torbert and of Bradley, Boyer, and Allen show that nonallelic loci are responsible for the defects of hemoglobins S and Ho-2 and that probably these loci are not closely linked. Our recombination data show that different chains carry the abnormalities of these hemoglobins, the β-chain of S and the α-chain of Ho-2, and that double heterozygosity probably results in production of four hemoglobins that are the four possible combinations of α_2^{A}, $\alpha_2^{\text{Ho-2}}$, β_2^{A}, and β_2^{S}. From these data we conclude that a pair of alleles at one locus controls the synthesis of α^{A}-chains and $\alpha^{\text{Ho-2}}$-chains and that a pair of alleles at a different locus controls the synthesis of β^{A}-chains and β^{S}-chains (Table 4). Furthermore each of

TABLE 4

PRODUCTS OF GENOTYPES WITH RESPECT TO α-CHAIN AND β-CHAIN LOCI

Genotype	Components		
	−2	0	+2
$\frac{\alpha^{\text{A}}\beta^{\text{A}}}{\alpha^{\text{A}}\beta^{\text{A}}}$	...	$\alpha_2^{\text{A}}\beta_2^{\text{A}}$	...
$\frac{\alpha^{\text{A}}\beta^{\text{A}}}{\alpha^{\text{A}}\beta^{\text{S}}}$	...	$\alpha_2^{\text{A}}\beta_2^{\text{A}}$	$\alpha_2^{\text{A}}\beta_2^{\text{S}}$
$\frac{\alpha^{\text{A}}\beta^{\text{A}}}{\alpha^{\text{Ho-2}}\beta^{\text{A}}}$	$\alpha_2^{\text{Ho-2}}\beta_2^{\text{A}}$	$\alpha_2^{\text{A}}\beta_2^{\text{A}}$ $\alpha_2^{\text{A}}\beta_2^{\text{A}}$	...
$\frac{\alpha^{\text{A}}\quad\beta^{\text{A}}}{\alpha^{\text{Ho-2}}\beta^{\text{S}}}$	$\alpha_2^{\text{Ho-2}}\beta_2^{\text{A}}$	 $\alpha_2^{\text{Ho-2}}\beta_2^{\text{S}}$	$\alpha_2^{\text{A}}\beta_2^{\text{S}}$

the four chains apparently is synthesized independently and in identical pairs. According to available familial studies the genes for hemoglobins C and E, which, like S, are abnormal in their β-chains,[4] behave as alleles of the gene for hemoglobin S.[6, 20] All these findings are consistent with the hypothesis that one locus determines the structure of the α-chain and a different locus, of the β-chain, and that mutation at either locus results in an abnormal hemoglobin.[21] An individual heterozygous for two abnormal genes at either of these loci would not have any hemoglobin A; conversely the absence of hemoglobin A in the presence of two ab-

normal hemoglobins would, according to this hypothesis, indicate that these abnormal hemoglobins carry their respective defects in the same chain and are controlled by allelic genes.[22] A doubly heterozygous individual would have four species of adult hemoglobin, which would separate electrophoretically into three or four electrophoretic components.[5]

Hemoglobins D_α and I are defective in the α-chain, and hemoglobins D_β and J are defective in the β-chain.[4, 16] Genetic control of these hemoglobins has not been established by familial studies; however, if the theory of control of α- and β-chains by nonallelic loci is correct, D_α, I, and Ho-2 are controlled by alleles at one locus, and D_β, J, and S, by alleles at another. Test of this theory requires familial as well as chemical data, for reliance on the latter alone may lead to an ambiguous result. For example, the presence of three or four electrophoretic components in the same individual probably would indicate double heterozygosity but would not distinguish between linkage or independence. Adjacent or closely linked loci would yield the same set of products as independent loci if the sites of synthesis of the chains are independent.

Atwater, Schwartz, and Tocantins[23] have recently reported familial and electrophoretic data consistent with nonallelic genetic control of hemoglobins C and G. The abnormal chain of hemoglobin G was not ascertained; however, a defect in the α-chain, as postulated by the authors, is in accord with their findings. Presumed double heterozygotes have four hemoglobins, A, C, G, and a hemoglobin with a mobility that corresponds to the composition, $\alpha_2^G\beta_2^C$. Nonallelic control of hemoglobins S and G was postulated earlier in a different kindred because an individual with hemoglobins S and G had a child with only hemoglobin A.[24] Neither this parent nor another member of the kindred with hemoglobins S and G had any hemoglobin A, and the defect of hemoglobin G was found in its β-chain.[25] These inconsistencies with the hypothesis of separate genetic control and independent synthesis of α-chains and β-chains apparently have been resolved, since re-examination has revealed that the parent in question actually does not have hemoglobin G.[26] It appears therefore that the two examples of hemoglobin G cited above are different molecular species with the same electrophoretic mobility. Shooter and co-workers[27] have reported a minor component, hemoglobin G_2, in a presumed homozygote for hemoglobin G. Hemoglobins G and G_2 of the sample examined by Shooter probably have the same abnormal α-chain and are analogues of hemoglobins A and A_2, which have the same normal α-chain.[28] The Ho-2 analogue of A_2 is not detectable in our electrophoretic patterns. If such a component were present, it probably would comprise less than 1 per cent of the hemoglobin of Ho-2 trait and would migrate between hemoglobins A and Ho-2. According to Jones and co-workers and Hunt hemoglobins A and F (fetal hemoglobin) have the same α-chain.[29, 30] The other chain of fetal hemoglobin is the γ-chain.[31] It is likely that a newborn infant with hemoglobin Ho-2 trait would have four hemoglobins, $\alpha_2^A\beta_2^A$, $\alpha_2^A\gamma_2$, $\alpha_2^{Ho\text{-}2}\beta_2^A$, and $\alpha_2^{Ho\text{-}2}\gamma_2$.

Summary.—We have shown that hemoglobin Ho-2 is abnormal in its α-chain and normal in its β-chain, in contrast to hemoglobin S, which is normal in its α-chain and abnormal in its β-chain. Familial data have shown that the loci which determine these two abnormal hemoglobins are genetically distinct and probably not closely linked. These results are consistent with the hypothesis that

different loci determine the structure of α-chains on the one hand and β-chains on the other. Presence of a normal and an abnormal allele at each locus probably results in four hemoglobins in the same individual, normal, doubly abnormal, and two singly abnormal. The fact that these four hemoglobins represent the four possible combinations of an abnormal and a normal pair of each of the two types of chains suggests that the chains are synthesized in identical pairs at independent sites.

We are indebted to Drs. Ernest W. Smith and Thomas B. Bradley for the samples of blood, and to Dr. T. Viswanatha for his collaboration on the chromatographic separations.

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† National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Maryland.

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*STUDIES ON THE EFFECT OF CARBON DIOXIDE ON X-RAY INDUCED CHROMOSOME ABERRATIONS IN TRADESCANTIA, I. DURATION OF THE PRETREATMENT EFFECT**

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Tradescantia microspores irradiated in one atmosphere of air plus one atmosphere of carbon dioxide show a higher frequency of chromosome aberrations than the air controls.[1-3] This observation has been reported on a variety of organisms.[4, 5] Except that carbon dioxide is an important modifying factor in the induction of chromosome aberrations by radiation, very little is known about the mechanisms involved. The present experiment was undertaken to determine whether the presence of carbon dioxide at the time of irradiation is a necessary condition for the enhancement of chromosome aberrations or whether pretreatment alone is sufficient.

Methods.—The irradiated material consisted of inflorescences from a clone of *Tradescantia paludosa* (Clone 5 of Sax) grown in nutrient solution. The inflorescences were placed in a Lucite chamber fitted with two-way valves and exposed to one atmosphere of air plus one of carbon dioxide for one hour in the dark. The chamber was then evacuated and refilled with air twice. At 1- to 40-minute intervals after the CO_2 treatment, the inflorescences were irradiated with 280 r of X rays (250 KVP, 30 ma, 4.5 mm Al HVL, 280 r/min). Controls were exposed to air alone and whenever possible were irradiated simultaneously with the experimental material. The temperature was maintained at 21–23° C. Throughout this paper the use of the term "CO_2 treatment" indicates a one-hour treatment with 1 atmosphere of air and 1 atmosphere of carbon dioxide.

The inflorescences were fixed four days after the treatment and propriono-carmine squash slides prepared. Those cells which were in metaphase at the time of fixation had been in interphase at the time of irradiation.[6] Microspores in metaphase were scored for dicentrics, centric rings, and interstitial deletions. All of these aberrations require two chromosome breaks for their formation. The slides were coded before scoring and all microscopic observation was made by one person.

Results and Discussion.—The results of two trials of the same experiment carried out four months apart, which were essentially the same, are summarized in Table 1 and Figure 1. The data indicate that treatment of the inflorescences with carbon dioxide before irradiation is effective in increasing the number of chromosome aberrations induced by X rays. The experimental results were tested by chi-square analysis and found to be significantly higher than the controls except for the group where the interval was 40 minutes. In the latter group neither chromosome aberra-

TABLE 1

THE DURATION OF THE CO_2 EFFECT ON CHROMOSOME ABERRATIONS INDUCED BY X-IRRADIATION IN AIR*
(280 r delivered in 1 min)

Time (min) between end of pretreatment and irradiation in air	Exchanges		Interstitial deletions		Total cells
	No.	Per cell	No.	Per cell	
0 (Irradiated in CO_2 + air)	451	0.657	325	0.473	687
1	267	0.608	188	0.428	439
5	225	0.563	172	0.430	400
10	95	0.528	98	0.544	180
15	325	0.518	264	0.420	628
20	331	0.525	261	0.414	631
40	174	0.423	120	0.292	411
Controls (no pretreatment)	361	0.402	292	0.325	898

* One-hour pretreatment with CO_2 and air was followed by an interval in air before irradiation.

tions nor interstitial deletions are significantly different from the controls. The CO_2 effect persists for at least 20 minutes after termination of the pretreatment. The effect of the CO_2 decreases rapidly during the first 10 minutes to a plateau maintained for about 10 minutes and then drops to control level by 40 minutes after the cessation of the treatment (Fig. 1). This initial steep decline and the

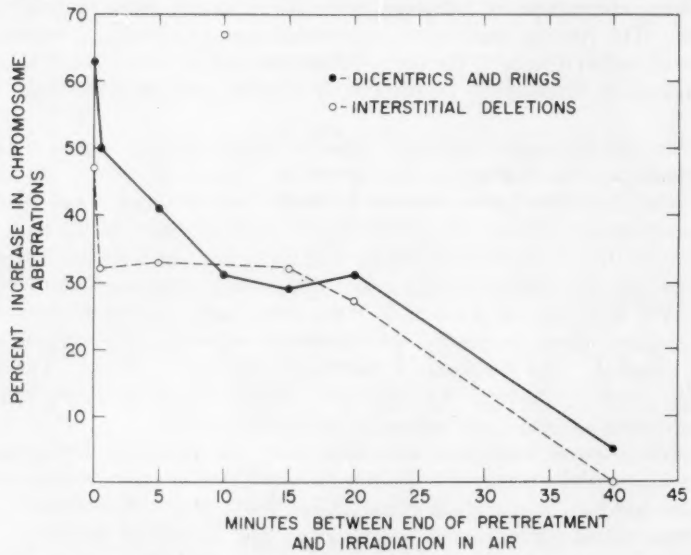

FIG. 1.—The effect of pretreatment with carbon dioxide on chromosome aberrations induced by 280 r of X-rays. The figures on the ordinate express the difference between treated and controls over controls (in per cent) as a function of time in air between the end of the pretreatment and irradiation in air.

subsequent plateau may be indicative of two systems involved in the carbon dioxide effect, but further speculation on this point would be premature.

Recent tests with gamma rays, X rays, 14 Mev neutrons and fission neutrons have indicated that the CO_2 enhancement of chromosome aberrations decreases with an increase in ion density of the radiation used and that significant enhance-

ment obtains only when sparsely ionizing radiations are used.[3] Since chromosome exchanges (dicentrics and rings) are caused mostly by two separate hits with gamma and X rays, but mostly by single hits with neutrons, it was proposed that the CO_2 enhancement applies mostly to two-hit types of aberrations. The hypothesis was advanced that the CO_2 treatment enhanced movement of broken chromosome ends resulting from the irradiation and thus favored new reunions rather than restitution. A significant increase in deletions was also observed indicating that less restitution was taking place. This hypothesis was based on indications that CO_2 decreases the viscosity of protoplasm.[7-11] It was, therefore, reasonable to envisage a greater movement of chromosomes in a medium of lowered viscosity. Furthermore, Northen (1940) has shown that this altered viscosity is not an irreversible state, but that the viscosity of the cell gradually returns to normal after the CO_2 treatment has been terminated. For *Spirogyra* cells, about 15 minutes sufficed for complete recovery of normal viscosity. The present results indicate that the effect of pretreatment with CO_2 on chromosome aberrations like the effect on viscosity is reversible and of relatively short duration.

The results of the present experiment seem to be in agreement with the above hypothesis. In order for the CO_2 pretreatment to be effective irradiation must follow within 20 minutes. This interval may be the period required for the cell to regain its normal physiological state or the period required for the nucleoplasm to return to normal viscosity. Unfortunately, little is known of the viscosity changes in the nucleoplasm. The extensive studies of Kamiya, Allen, and Price[8, 9] concerning the energetics of protoplasmic streaming and viscosity have been performed on the cytoplasm of the slime mold. The fact that CO_2 treatment decreases the viscosity of the cytoplasm of plant cells suggests it may do the same to the nucleoplasm.

Studies of O_2 uptake after CO_2 pretreatment: Several studies have suggested that certain agents affecting respiration of tissues are capable of preventing restitution and thereby keep breaks open to form aberrations over extended periods.[12-14] Since CO_2 is implicated in the respiration of plants and there exist various mechanisms and directions by which excess CO_2 might effect respiration,[15-17] it seemed desirable to determine the oxygen uptake rate of the *Tradescantia* inflorescences at various times subsequent to a carbon dioxide treatment. The oxygen uptake experiments were carefully controlled so as to duplicate the irradiation studies. Two groups of *Tradescantia* inflorescences were placed in a Warburg apparatus to determine the normal oxygen uptake. After the control level had been established, one group was placed in a chamber in 1 atmosphere of air and an additional atmosphere of carbon dioxide was introduced. The experimental chamber was kept in the dark for one hour with the air control. After the pretreatment period the chamber was evacuated twice and refilled with air, just as for the radiation experiments. The flask containing the CO_2 treated inflorescences was removed and placed in the constant temperature bath (21°C) with the control group. A four-minute period was allowed for temperature equilibration and the readings of oxygen uptake, in the light, were taken at regular intervals for one hour. The results are shown in Figure 2.

The results of the oxygen uptake studies indicate that a very dramatic increase in O_2 uptake occurs in the plant tissues either during or within four minutes after

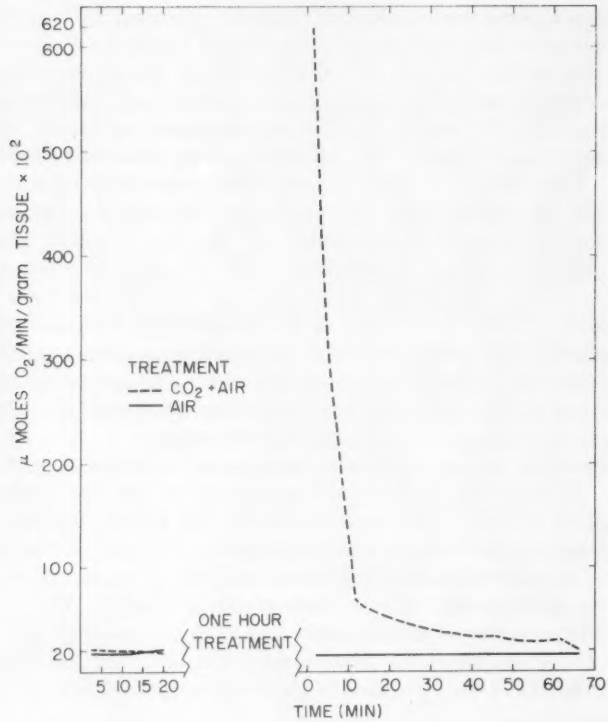

FIG. 2.—Effect of treatment with carbon dioxide on subsequent oxygen uptake in air. The rates were measured before and after the indicated treatment.

they are removed from an environment of CO_2 and air. The oxygen consumption falls very rapidly for about 10 minutes after treatment but remains above the control levels for at least one hour. This can be related to the results of the chromosome aberration studies. The effect of pretreatment with CO_2 on chromosome aberrations is greatest if the irradiation quickly follows the pretreatment. The oxygen uptake during the first four minutes after treatment must be very high, judging from the shape of the curve and the fact that after four minutes it is still 30 times higher than the controls. Chromosome aberrations are 30 per cent higher than the controls during the period 10 to 20 minutes after the pretreatment and the oxygen uptake during this same period is still *twice* that of the controls. The difference between treated and control inflorescences in the oxygen uptake experiments is much greater than the differences observed in the chromosome aberration experiments, especially during the first few minutes. However, the shapes of the curves and the time required for both phenomena to return to control levels are similar.

The carbon dioxide pretreatment is enhancing the radiation-induced chromosome aberrations while the oxygen consumption of the tissues is much higher than the controls. This relationship may be coincidental. It is possible that restitution

of chromosome breaks is not occurring when irradiation follows a CO_2 treatment despite the oxygen uptake rate of the tissues. Perhaps this high O_2 consumption represents the repayment of an oxygen debt incurred during the one-hour CO_2 treatment and that only after the debt has been paid is energy available for chromosome restitution. Also, carbon dioxide treatment may uncouple oxidation from phosphorylation and the high oxygen uptake would reflect an adjustment of the tissues to maintain the level of phosphorylation. Such a mechanism would relate the CO_2 effect to some of the chemicals which have been postulated to inhibit restitution of chromosome breaks.[12-14] In any case, if chromosomes broken by irradiation are not restituting and are moving apart, then when energy subsequently does become available, the probability of forming an aberration is greater than of restitution to the original configuration.

On the other hand, CO_2 may exert its effect entirely by decreasing viscosity, thus enhancing chromosome movement. At the present time, it is difficult to state which portion of an intricate series of cellular changes best accounts for the enhancement of radiation-induced chromosome aberrations caused by pretreatment with carbon dioxide.

Summary.—The pretreatment of *Tradescantia* inflorescences with one atmosphere of CO_2 plus one of air is effective in increasing the number of chromosome aberrations induced by X-rays by about 1.6 times. Separating the CO_2 pretreatment and radiation by increasing periods in air has shown that the CO_2 effect persists for about 20 minutes. After this period the aberration frequency returns to control levels. Following the CO_2 treatment the oxygen uptake rate of the tissues is greatly increased. There is reason to believe that changes in nucleoplasmic viscosity and oxidative metabolism may alter the frequency of chromosome aberrations induced by radiation.

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*BIOLOGICAL CLOCKS IN MEDICINE AND PSYCHIATRY: SHOCK-PHASE HYPOTHESIS**,†

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Communicated September 23, 1960

Biological clocks in medicine and psychiatry we think of as devices of the body that keep time with relative independence of external conditions and events: each in its own units and with varying degrees of accuracy.

What are these clocks? And where are they located? By all odds the best-known example is the ovarian clock, which for several decades of a woman's life determines the time of ovulation, usually at about 28-day intervals.

What other clocks are there? Why aren't they more readily apparent? What makes them run? What part do they play in our lives? Of what interest are they to medicine in general and psychiatry in particular?

Study of these clocks produces new perspectives on the normal and abnormal functioning of various organs of the body, such as peripheral organs, endocrine glands, and the brain.

It is true that normal human beings give little indication of possessing biological clocks—other than the 24-hour clocks of men and women and the 28-day menstrual clocks of women. But many other timed mechanisms are present, as becomes unmistakably clear under certain abnormal conditions.

These clocks manifest their existence through the appearance of one or more of a number of physical and mental symptoms. Let me give a few examples: first, of clocks manifesting themselves through primarily physical symptoms; and second, of others that become evident through primarily mental or emotional symptoms. (I shall in each case present them in order of the lengths of the units in which the clocks measure time, beginning with the shortest intervals we have detected so far.)

CLOCKS THAT MANIFEST THEIR PRESENCE THROUGH PRIMARILY PHYSICAL SYMPTOMS

Evidence for the existence of a clock that measures time in the shortest units (12 hours) is presented in the temperature chart of a 19-year-old girl in Figure 1.[1] Ordinates show body temperature readings taken at frequent intervals throughout the day and night. Peaks of 104.2–105.3°F were reached twice every 24 hours. With ascending temperatures the patient was increasingly ill; once the peak had been passed she felt a remarkable relief from symptoms. Blood and other studies failed to explain the fever.

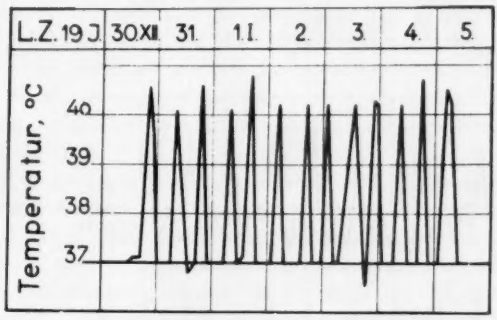

FIG. 1.—Hourly temperature readings showing 12-hour spikes of body temperature in a 19-year-old girl (Hitzig, 1955). These peaks came in a series of 20–28 fairly regular intervals of 5 months. It is noteworthy that these temperature cycles had been present in this patient from early childhood; further that almost identical cycles subsequently appeared in her daughter at the age of 9 months and were still definitely present at the age of 6 years—at the time of the publication of Hitzig's report. During the intervals between each series of temperature peaks, both mother and child were entirely free from all abnormal symptoms.

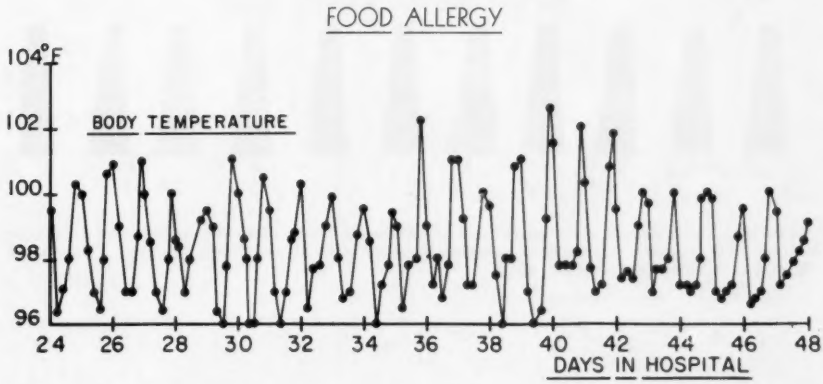

FIG. 2.—Graph showing part of daily records (24–48th day) of 130-day stay in hospital of an 18-year-old girl suffering from food allergy and showing 24-hour cycles. Rowe's cereal-free elimination diet, started on the 122nd day of the patient's stay in the hospital, promptly interrupted the 24-hour fever cycles. (Redrawn from Rowe, 1948.)

The presence of a clock that measured time in units of 24 hours may be seen in the temperature chart of an 18-year-old girl in Figure 2.[2] This patient suffered from a food allergy. Ordinates show body temperature recorded at frequent intervals during the day and night. Body temperature fluctuated at 24-hour intervals not only far above but far below the normal level. During periods of fever the patient experienced cramping in the mid-abdomen and in the rectum; at other times she was free of discomfort.

Evidence for the existence of a clock that measured time in units of 48 hours is presented in the daily records of body temperature and pulse rate of an 8-year-old boy in Figure 3.[1] Since the age of 4 months this boy had shown attacks of fever,

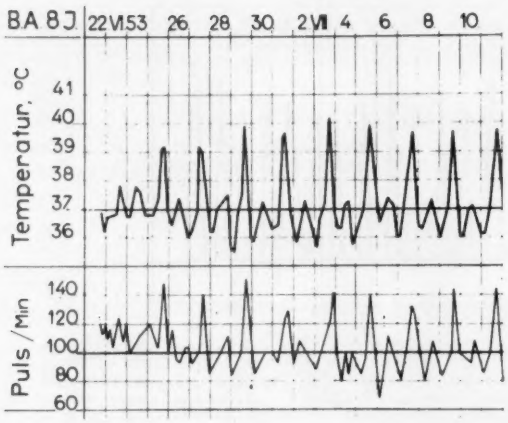

FIG. 3.—Graph showing daily records of body temperature and pulse rate of 8-year-old boy (Hitzig, 1955).

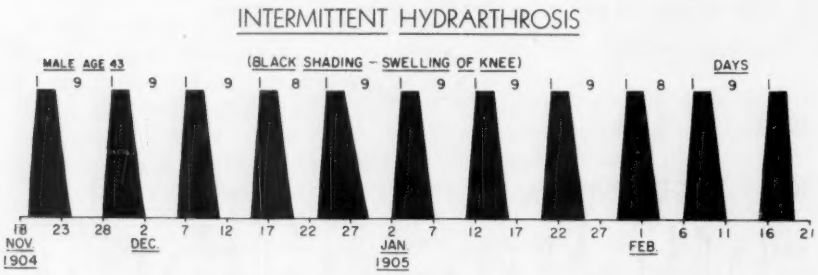
INTERMITTENT HYDRARTHROSIS
MALE AGE 43
(BLACK SHADING – SWELLING OF KNEE)
DAYS
9 9 9 8 9 9 9 9 8 9
18 NOV 1904
23 28 2 DEC. 7 12 17 22 27 2 JAN 1905 7 12 17 22 27 1 FEB. 6 11 16 21

FIG. 4.—Daily records showing periods of swelling of knee in a 43-year-old man with intermittent hydrarthrosis. Lengths of intervals between attacks are shown in days. (This 95-day chart was redrawn from Garrod, 1910.)

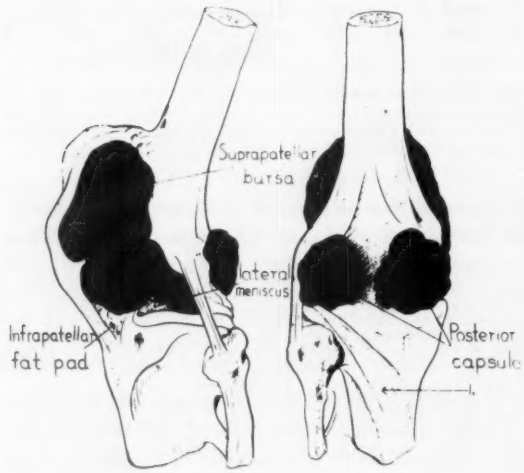

FIG. 5.—Drawings showing the synovial spaces (solid black shading) of the knee. (Modified from Brantigan and Voshell, 1941.)

severe pain in head and chin, polyuria, neutropenia, every 48 hours and lasting 3–5 hours. The original attack occurred after a period of high fever subsequent to small-pox vaccination. Between attacks the patient played and was free of all disturbances. From the presence of an enlargement of the third ventricle, polyuria, and other symptoms, Hitzig concluded that the patient probably suffered from a lesion in the diencephalon.

The existence of a clock that measured time in still longer units (9 days) was manifested in a 43-year-old man by periodic swelling of the right knee (Fig. 4).[3] Solid black areas indicate times during which the right knee was swollen. Intervals between onsets of succeeding attacks are shown in days. In each attack the knee remained swollen 3–5 days; during the remainder of the cycle there was no evidence of abnormality. The synovial spaces that become filled with fluid during episodes of hydrarthrosis are illustrated in the drawing in Figure 5.[4] Many of these patients state that at the start of an attack they have the sensation of water literally rushing into the knees.

Evidence for the existence in one individual of two independent clocks that measured time in units of 7 or nearly 7 days is shown in the record of a 47-year-old man also with intermittent hydrarthrosis (Fig. 6).[5] In this case the chart gives

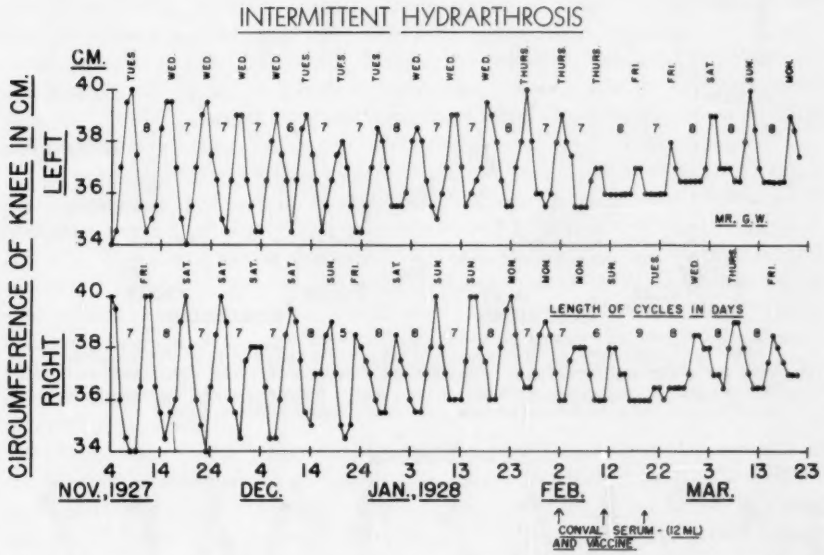

FIG. 6.—Daily records of circumferences of the right and left knees of a patient with intermittent hydrarthrosis. Ordinates give circumference of each knee in centimeters. (Redrawn from Baker, 1929.)

daily measurements of the circumference of each knee in centimeters for a period of 140 days. In many instances the swelling in one knee was at a maximum while it was at a minimum in the other. Thus, at the start of the record, maximum peaks came on Tuesdays and Wednesdays in the left knee and on Fridays and Saturdays in the right.

The presence of a clock that measured time in still longer units (17–19 days) was manifest in the blood count of a 41-year-old woman suffering from cyclic agranulocytosis as seen in Figure 7.[6] The number of polymorphonuclear cells fluctuated between 4,000 and zero every 17–19 days. Lip and cheek ulcers appeared during each cycle just before the number of neutrophils, important defenders of the body against infection, reached these zero levels. The patient experienced malaise coincident with the agranulocytosis. There is evidence here also of the existence of a second clock, the menstrual clock, in this case measuring time in units of 34 days and entirely independently of the other.

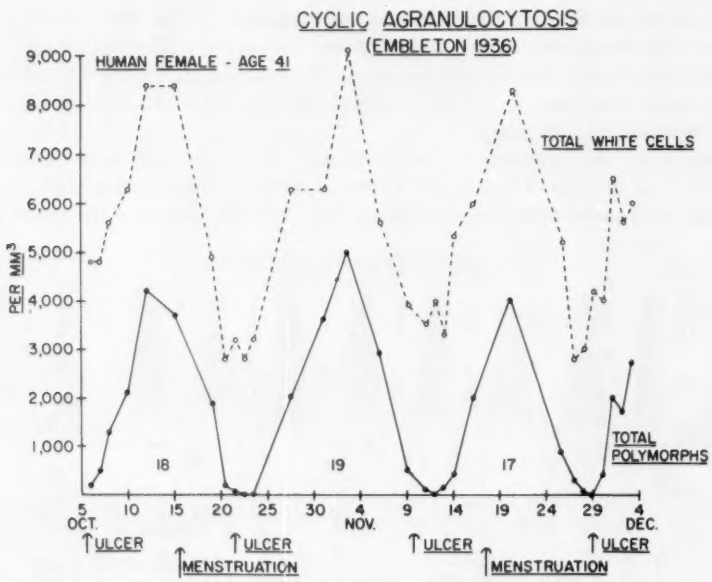

FIG. 7.—Graph showing daily records of total white cells and polymorphonuclear cells per cubic mm of blood in a 41-year-old woman with cyclic agranulocytosis. The lengths of the cycles are indicated in days. Arrows indicate appearance of mouth ulcers and of menstrual periods. (Redrawn from Embleton, 1936.)

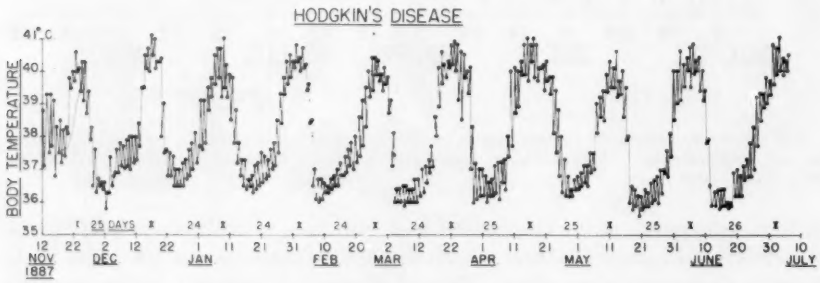

FIG. 8.—Graph showing daily body temperature records of a 19-year-old boy with Hodgkin's disease. The numbers over the base line give the lengths of the temperature cycles in days. (Redrawn from Ebstein, 1887.)

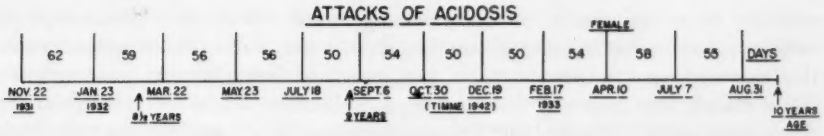

FIG. 9.—Graph showing record of attacks of acidosis in a young girl and length of intervals between attacks. (Chart made from data taken from paper by Timme, 1942.)

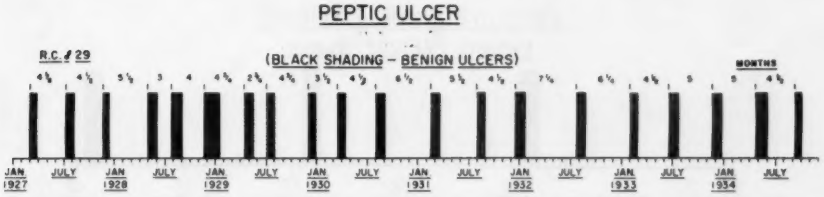

FIG. 10.—Graph for 29-year-old man showing record of appearance of recurrent duodenal ulcers. Solid black shading indicates appearance and duration of ulcers. Numbers at the top show intervals in months between onset of successive attacks. (Redrawn from Jahiel, 1953.)

Evidence for the existence of a clock that measured time in still longer units (24, or nearly 24, days) is shown in the temperature chart of a 19-year-old boy with Hodgkin's disease (Fig. 8).[7] This chart gives daily morning-evening temperatures over a 240-day period. During the periods of elevated temperatures the patient complained of tiredness, headache, loss of appetite, and abdominal pain and showed increased pulse and respiratory rates, loss of weight, splenomegaly, and lymphadenopathy.

The existence of a clock that measured time in units of 52 or nearly 52 days is demonstrated in the chart in Figure 9 which shows the occurrence of attacks of acidosis in a young girl, aged $8^1/_2$ years at the time of the first attack.[8] Beginning at an age of $1^1/_2$ years this child showed definite symptoms of pituitary, thymic, and calcium disturbances, growth abnormalities, and attacks of allergy consisting of skin eruption, asthma, gastrointestinal upsets, and fever. Later, acidosis became the chief symptom of the attacks which persisted up to her 13th year. Treatment with pituitary substance, thyroid powder, and calcium lactate, helped to reduce the number and severity of attacks. Two other of Timme's patients also had attacks of 50–60 days in length.

The presence of a clock that measured time in still longer units ($4^1/_2$ months on the average) is demonstrated in the chart in Figure 10 which shows the occurrence and duration of attacks of benign duodenal ulcers over an 8-year period in a man who was 29 years old at the onset of the illness.[9] During these attacks, each lasting 10–15 days, the patient suffered from postprandial pain.

CLOCKS THAT MANIFEST THEIR PRESENCE THROUGH PRIMARILY MENTAL AND EMOTIONAL SYMPTOMS

Now let us turn to examples of clocks that manifest their presence through primarily mental and emotional symptoms. Evidence for the presence of a clock that measured time in units of 24 hours was seen in a 28-year-old woman suffering from Parkinsonism subsequent to an attack of encephalitis early in childhood.[10] During

each day up to nine o'clock in the evening the patient was bed-ridden, unable to walk, or to feed herself because of a marked rigidity and tremors of her legs and arms. Her handwriting was indecipherable, her speech unclear; but she was euphoric. Quite sharply near nine o'clock in the evening she showed a sudden change in her whole personality. Rigidity and tremors disappeared; she was able to walk, feed, and otherwise take care of herself; her speech and handwriting became perfectly clear; the euphoria disappeared to leave in its place a state of apathy. Figure 11

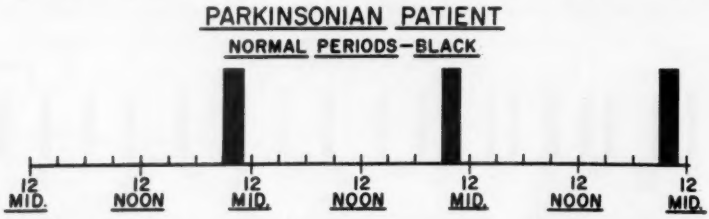

FIG. 11.—Graph made from the clinical report (Leonard, 1931) of a 25-year-old woman with Parkinsonism. The black shaded areas lasting 2–3 hours each 24 hours are periods during which the patient was able to walk, feed herself, talk and write clearly, but was apathetic.

shows a graph of these changes. These 24-hour cycles were present during the nine-year observation period in the hospital.

The presence of a clock that measured time in units of 48 hours is evidenced in the excerpts of a so-called behavior chart (Fig. 12) kept by trained nurses for a

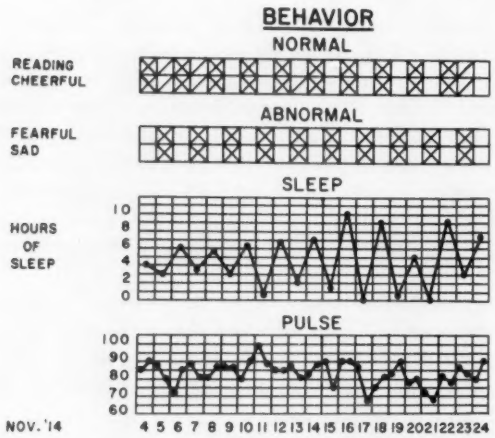

FIG. 12.—Part of behavior chart of 64-year-old woman showing presence or absence of normal and abnormal behavior and mood; also body temperature and pulse rate. A 48-hour cycle is clearly shown in behavior and mood, hours of sleep, and pulse rate. (Richter, 1938.)

64-year-old woman from the Phipps Clinic.[11] The two top rows of squares are marked for normal behavior and mood: reading, cheerful; the two squares in the second row are marked for abnormal behavior: fearful, sad (actually suicidal). Here we see a 48-hour cycle, 24 hours of normal and 24 hours of abnormal behavior. This same cycle is clearly reflected in hours of sleep and definitely, but less clearly, in pulse rate. In this case transition from one phase to the other was very sharp—

often occurring within a few minutes—and at almost exactly the same time on successive nights. This is the same cycle seen in the patient with primarily physical symptoms whose record is shown in Figure 3.

Evidence for the existence of a clock that measured time in slightly longer units (5 days) comes from another Phipps Clinic patient, a 30-year-old woman diagnosed as "constitutional inferiority with hypomaniac features."[12] In Figure 13, which

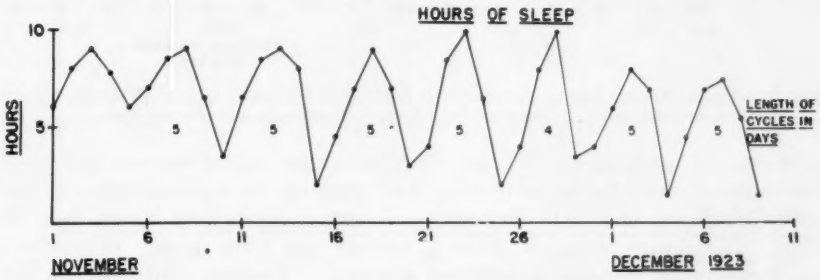

FIG. 13.—Graph showing daily record of hours of sleep of a 30-year-old woman diagnosed as "constitutional inferior with hypomanic features." (Richter, 1934.)

shows hours of sleep, we see the presence of a clock that measured time in units of 5 days. One to two days of excitement and sleepless nights were followed by periods of 2–3 days of sluggish behavior and nights of normal sleep.

The presence of a clock that measured time in still longer units (19–24 days) is evidenced in the daily records of a 22-year-old male catatonic-schizophrenic patient with recurrent periods of excitement (Fig. 14).[13] The top graph repre-

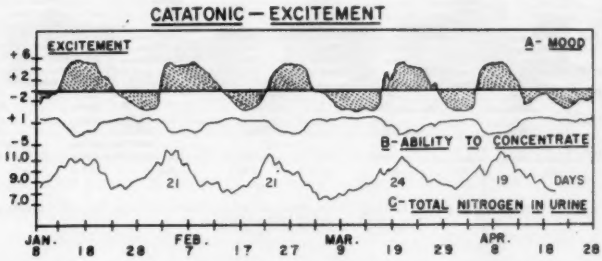

FIG. 14.—Graph showing daily records for 22-year-old male catatonic-schizophrenic patient with periodic excitement. (Redrawn from Gjessing, 1936.)

sents estimates of mood; the second, a measure of the patient's ability to concentrate; the third, total urinary nitrogen. The record extends over a 110-day period. Throughout the entire time, the patient was kept in bed and on a constant diet. This patient was alternately wild and excited, so much so that he had to be forcibly restrained, and then normal or slightly depressed. Here again the transition between the two phases occurred within a very short period. During the excited phases the patient had difficulty in concentration. Sinusoidal cycles of the same lengths appeared in total nitrogen metabolism.

Evidence for the existence of a clock that measured time in units of 40 days may be seen in the excerpt from the daily behavior chart for a Phipps Clinic patient,

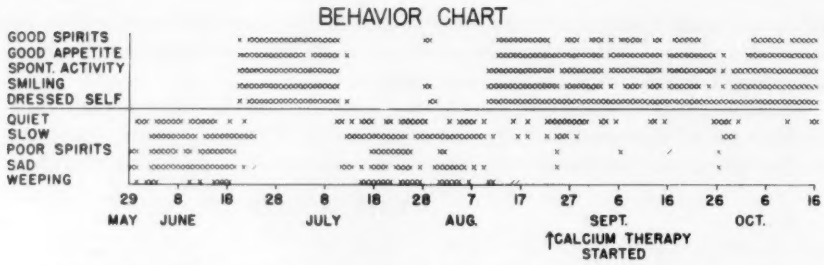

FIG. 15.—Graph showing part of daily behavior chart of 56-year-old woman with apparent parathyroid deficiency. (Redrawn from Richter, Honeyman and Hunter, 1940.)

a 56-year-old schoolteacher (Fig. 15).[14] Markings for normal behavior and mood are indicated above the center dividing line; markings for depressed behavior and mood, below the line. The depressed and normal phases both lasted about 20 days. The change from one phase to another was quite sharp. This patient suffered from an apparent parathyroid deficiency. Treatment with calcium lactate solution and AT_{10} (Dihydrotachysterol) corrected the calcium deficiency and abolished the attacks.

The existence of a clock that measured time in units of 52, or nearly 52, days is demonstrated in the excerpt of the daily behavior chart of a 14-year-old boy (Fig. 16).[15] Markings for manic behavior and mood are shown on the top third of the

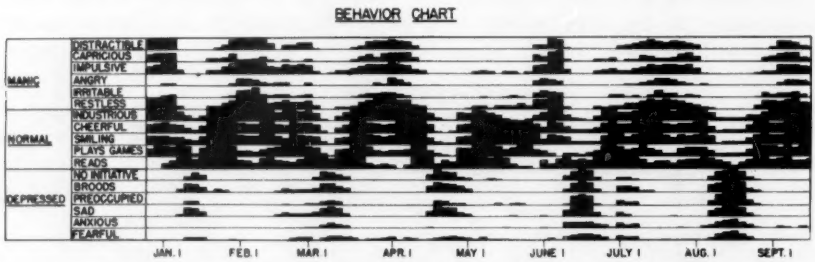

FIG. 16.—Part of behavior chart showing daily record of behavior and mood of 14-year-old boy. (Redrawn from Rice, 1944.)

chart; for normal behavior and mood, on the middle third; and for depressed behavior and mood, on the bottom third. At the age of 14 years this boy suddenly began showing circular attacks that followed a succession of changes in behavior and mood from manic to normal, to depressed, to normal, to manic, etc. He was observed in the Phipps Clinic for $8^1/_2$ months and in another hospital for 9 months, at the end of which time the attacks still occurred with the same regularity. It will be noted that in this patient with primarily mental and emotional disturbances, the lengths of the cycles were exactly the same as those of the three patients with primarily physical attacks described by Timme.[8]

The presence of a clock that measured time in much longer units, 15–17 months, was seen in a manic-depressive patient during 6 years of her life spent in the Municipal Hospital in Vienna (Fig. 17).[16] The ordinates give weight in kilograms,

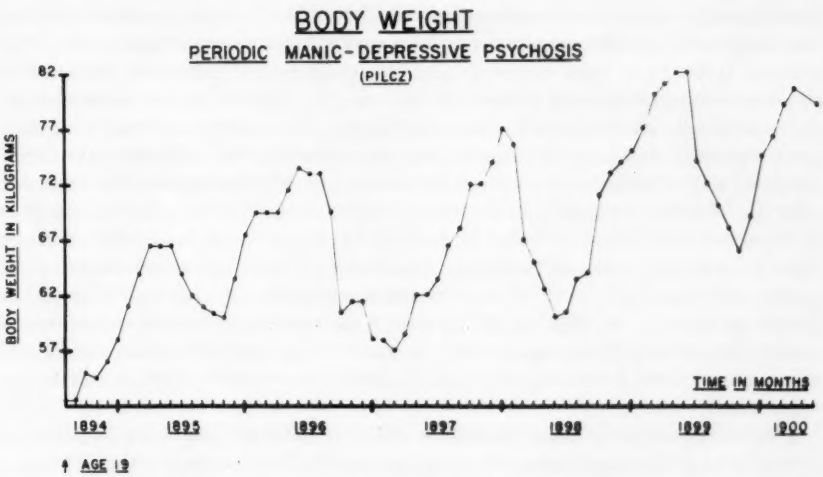

FIG. 17.—Graph showing body weight at monthly intervals of a manic-depressive patient in the Municipal Hospital of Vienna. (Redrawn from Pilcz, 1901.)

abscissas time in months. During manic periods the patient lost weight, which she more than regained in each successive period of normal or depressed behavior.

CHARACTERISTICS OF BIOLOGICAL CLOCKS

Over the course of years I have collected records of more than 500 patients whose symptoms have occurred with clock-like regularity. Some of the records came from the medical and psychiatric departments of the Johns Hopkins Hospital, some from other hospitals, but most of them came from the literature. I have combed the world literature of medical and psychiatric publications from the XVIIth Century to the present time for reports of cyclic clinical phenomena, particularly for charts showing actual daily measurements. This is the first time all this material has been correlated from the point of view of a biologist rather than that of a clinician, and with special emphasis on the cycles themselves, their duration, and other characteristics. Most records were obtained during the past century and the early part of the present century, when medical men, including psychiatrists, were concerned with obtaining careful detailed records of the clinical course of illnesses rather than with effects of various forms of treatment.

From the standpoint of research it is to be regretted that our increased knowledge and greatly improved means of treatment have led to the elimination of much of the characteristically detailed early reports. Some of the most significant recent records on psychiatric patients were made by a biochemically-minded psychiatrist, Gjessing in Norway, and by his followers in Germany, the United States, and New Zealand. Gjessing kept his patients under strictly controlled situations and kept daily records of many different metabolic and mental functions over long periods of time. Many good records of patients with primarily physical records were made or collected by Reimann.[17]

That we deal here with true "internal" clocks is shown by the fact that the various

physical and mental manifestations recurred with great regularity, in most instances over long periods of time despite marked changes in external conditions to which the patients must have been exposed—such as temperature, humidity, barometric pressure, and other external influences—and despite reactions to the many day-to-day emotionally charged events and occurrences. The attacks occurred with such great regularity that in many instances the person was able to predict months ahead the exact day of an attack—in some instances, not only the day but the hour and even the minute. One man, an insurance company officer, who had alternate days of depressed and normal behavior, showed me his appointment book with alternate days crossed off his calendar many months ahead. Under controlled conditions of a clinic this man had shown 48-hour cycles of metabolic function as well as of behavior and mood. At Cambridge University the schedule for soccer games over a season was specifically arranged so that no game fell on days when their best player, who suffered from hydrarthrosis of an injured knee every 9 days, would be incapacitated.

These various clocks measure time in different units ranging from 12 hours to several years. In many instances each unit consists of two phases; either the one is abnormal and the other normal, or they may both be abnormal. Either may be longer; the transition from one phase to the other is often strikingly abrupt. The relative lengths of the two phases, as well as the nature of the transition from one to the other, may vary from person to person. In some instances the cyclical curve may be sinusoidal. In any one patient the length and characteristics of the cycles tend to remain exactly the same.

These biological clocks may manifest themselves with equal frequency in males and in females and they may appear at any age from shortly after birth to old age. They may persist for months, years, or a lifetime, or they may be evident for only short periods, either to disappear forever, or to return again later.

Further, these biological clocks may manifest themselves with equal frequency in patients whose symptoms are primarily physical and those with primarily mental or affective symptoms—and the units in which their clocks measure time have much the same range, 12 hours to a year or more. Not only do the units have much the same ranges but in a number of instances so far they have been found to have the same lengths. This is most notably true of patients with 48-hour clocks; two such records were presented at the beginning: one showing primarily physical symptoms (Fig. 3), the other primarily mental symptoms (Fig. 12). Instances were also presented in which clocks that measured time in units of approximately 52 days were present in both groups of patients (Figs. 9 and 16 respectively). A number of other such instances are at hand. The further analysis of our data will undoubtedly bring out still others.

In every instance in which examinations of physical functions were made in patients with primarily mental and emotional symptoms, definite evidences were also found for the presence of physical symptoms. This indicates that in the one group clocks manifest their presence by physical signs; in the other, through both physical and mental signs and symptoms.

It may be noted that many psychiatrists have long denied the existence of inherent clock mechanisms in their patients in the belief that recurrences of abnormal mental and affective states result from reactions to recurring emotional stimuli and

disturbing life situations. The close similarity between the clocks of the two types of patients—established here for the first time—indicates that both kinds depend on the operation of inherent cyclic mechanisms.

It is significant that with few exceptions the clocks may manifest themselves through various forms of periodic pathological changes without leaving any detectable lasting physical or mental effects even after years or an entire lifetime of recurrences. In other words, between attacks the individual remains normal, thus providing his own normal baseline for comparison of changes that occur during attacks.

It must be mentioned here that thorough examinations made in many of the patients have failed to reveal the presence of any infectious organisms, the cyclic multiplication of which could account for the periodic changes. It is true that infectious diseases have been found in some of these patients with periodic illnesses, but elimination of the infection did not interfere with the cycles.

It may be mentioned at this point that the results of studies on the experimental production of abnormal cycles of behavior and metabolism in animals carried on since 1921 have helped to provide a more general perspective on the biology of clock phenomena. Results of these studies were summarized in my Salmon Lectures and will be referred to here only in passing.

NONSPECIFIC SYMPTOMS OF PRESENCE OF CLOCKS

The presence of clocks may be manifested by cyclic changes in almost any organ of the body, as may be seen in Table 1 which lists the organs that in one patient or another have been reported to show cyclic changes—joints, bone marrow, lymph glands, stomach and duodenum, etc., and the respective clinical manifestations; or by cyclic changes in almost every mental or emotional function—stupor, depression, elation, excitement, etc., as may be seen in Table 2, which lists the various symptoms that have been reported to occur in one or the other of patients with periodic psychiatric illnesses.

TABLE 1

ORGANS AFFECTED IN PERIODIC ILLNESSES, AND ASSOCIATED CLINICAL MANIFESTATIONS

Organs	Clinical manifestations
Joints (synovial spaces)	Intermittent hydrarthrosis, pain
Bone marrow	Cyclic neutropenia, agranulocytosis (ulcers) thrombocytopenia (bleeding, reticulocytopenia, anemia)
Lymph glands	Cyclic lymphocytosis, monocytosis, fever, Hodgkin's
Stomach and duodenum	Cyclic peptic ulcers, vomiting, diarrhea, fever
Peritoneum	Benign paroxysmal peritonitis, pain, fever
Salivary glands	Cyclic excessive secretion
Sweat glands	Cyclic excessive sweating
Spleen	Cyclic neutropenia, fever
Kidney	Cyclic hematuria, oliguria, polyuria, fever
Muscles	Familial periodic paralysis
Eyes	Cyclic iritis, polyserositis
Skin	Cyclic purpura, urticaria, angio-neurotic edema, erythema, fever
Brain	Cyclic epilepsy, hypo- and hyperthermia, insomnia, hypersomnia, headache, migraine

It must now be pointed out that some of these symptoms may be specific for a certain clock unit or for a definite disease, others may not be. The outstanding example of nonspecific physical manifestations is fever. Thus the chart in Figure 18 shows that fever may be an indicator of the presence of any one of the clocks that

TABLE 2

MENTAL AND EMOTIONAL STATES IN PERIODIC ILLNESSES

Stupor	Hallucination
Depression	Hypo-or hypersomnia
Elation	Bulimia
Excitement	Dipsomania
Mania	Actual changes of personality
Paranoia	Hypochondriasis

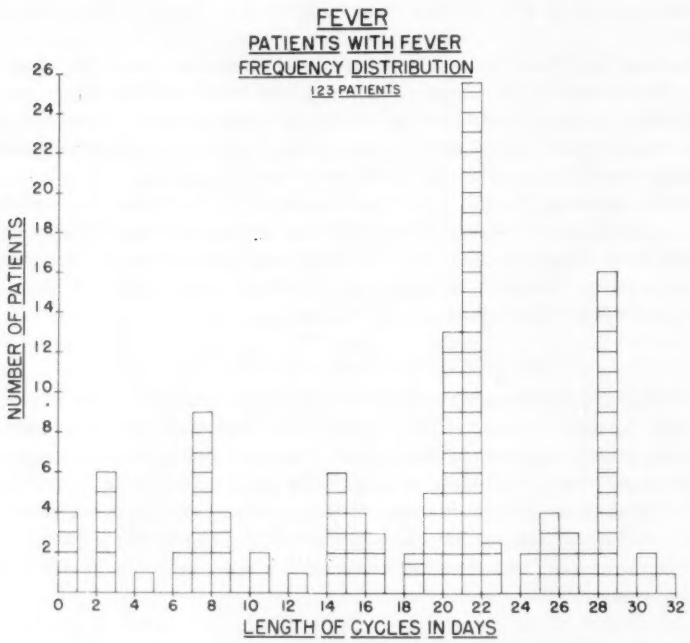

FIG. 18.—Distribution chart from 123 patients in which fever was one of the outstanding symptoms. Abscissas show length of fever cycles in days; ordinates number of patients with each length of cycle.

measure time in units ranging from 12 hours to 30 days, or more. This chart shows the average length of cycle for each of the 123 individuals in whom the presence of a clock was manifested by periodic peaks of body temperature. Abscissas give the length of the units in days; ordinates show the number of individuals having each length of cycle.

It is noteworthy that the fever chart shows maxima of numbers of individuals at 7 days and multiples of 7, namely 14, 21, and 28 days. The possible significance of this remarkable pattern will be discussed at another time.

Data for pulse rate and number of leucocytes are not available for the same large number of individuals but, since in all instances in which data are available, an increased pulse rate and leucocytosis so closely followed fever, it is likely that these two functions follow the same pattern as in Figure 18.

It is possible that most mental and affective symptoms may be nonspecific;

thus stupor may be a symptom of a 48-hour or of a 43-day clock; depression maybe a symptom of a 48-hour, or of a 15-month clock, etc.

How the mental symptoms come to be nonspecific can be learned from histories of patients with a 48-hour clock, 24 hours each of a "good" and "bad" day. These histories show that a fairly close relationship exists between behavior and mental status on "good" and "bad" days and the more or less chronic behavior and mental conditions present before the superimposition of the 48-hour cycle. Thus, in a normal person the presence of the 48-hour clock may manifest itself only through physical symptoms, cycles of body temperature, pulse rate, edema, etc., whereas a schizophrenic may show marked schizophrenic reactions on the "bad" day, reduced or no schizophrenic symptoms on the "good" days, and a depressive patient may be deeply depressed on his "bad" day, cheerful, communicative, etc. on the "good" day.

Of special interest here is the fact that in patients suffering from a fairly constant state of psychotic or neurotic symptoms, the appearance of the clock suffices not only to break up the mental and affective states into 48-hour periods, but to return the patient to a normal or nearly normal condition in the 24-hour period of the cycle.

A full knowledge of the mechanisms of the 48-hour clock—how the clock works—would throw light on the control of the great variety of psychotic and other states which it is able to modify in such remarkable fashion.

We may conceive that fever, leucocytosis, and other nonspecific symptoms have the same relation to the internal clocks that the hands have for an ordinary clock—they are simply indicators of the clock's running.

SPECIFIC SYMPTOMS OF THE PRESENCE OF CLOCKS AND POSSIBLE SITES OF CLOCK MECHANISMS

Now let us turn to instances in which a symptom or illness is associated with a definitely limited range of clock units, instances in which the symptoms may be a part of the clock mechanism itself. I shall start with intermittent hydrarthrosis, a condition in which we have records for the largest number of patients and also an illness which, as will be seen, offers almost ideal experimental conditions for our purposes: there is no fever, no leucocytosis, no changes in pulse rate, no mental or emotional symptoms—in most instances swelling of the joints constitutes the sole abnormal symptom. In any one patient the lengths of the cycles of swelling of a joint tend to remain so constant over periods of months or years that we are able to speak of a characteristic rather than an average length of cycle. Figure 19 presents the distribution curve of the characteristic lengths of cycles for 128 patients with intermittent hydrarthrosis—all of the patients for whom we have definite records of lengths of cycles. The ordinates record the number of patients showing characteristic cycles of each duration as measured in days. As can be seen, the lengths of these cycles almost all fall within a very narrow range, 7–14 days inclusive. Thus, in keeping with our thinking in the cases already discussed, we assume that these patients harbour clocks that keep time in units coming within this range.

The striking facts concerning the periodicity in this particular syndrome are that although in most patients with multiple joint involvement, swelling occurs at the same time in all joints—that is, all joints have the same lengths of cycles and

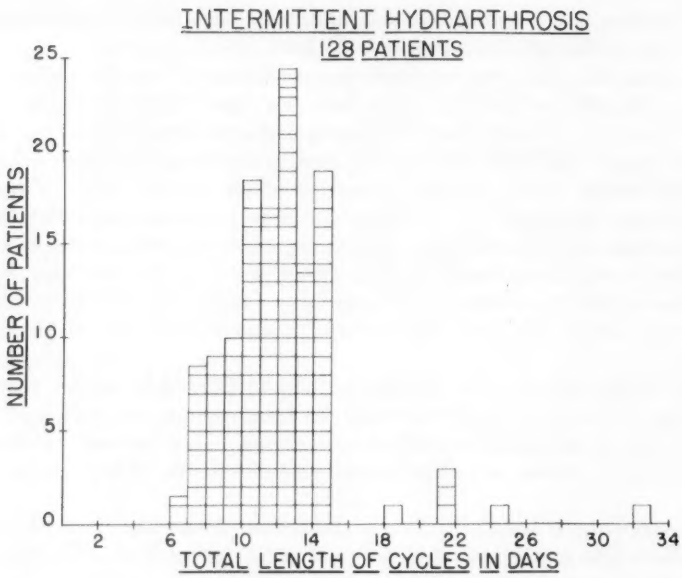

FIG. 19.—Distribution chart for 128 patients with intermittent hydrarthrosis. Abscissas give lengths of cycles in days; ordinates, number of patients with each length of cycle. In most instances the lengths of cycles for any one patient remained the same over long periods of time with only an occasional variation of a day or two.

all are in phase, in some patients, as we have seen, the lengths of cycles may be the same in two or more joints, but the swelling may reach maxima at different times in the respective joints. That is, although the cycles have the same lengths, they are out of phase. In still other patients with multiple joint involvement, the cycles of swelling in three or more joints may not only be out of phase but have different lengths; that is, in one joint it may be 8 days, in another 9 days, in another 10 days. This means that the joints may function quite independently of one another —still, however, within the range of 7–14 days. These observations make it unmistakably clear that the joints function not only independently of one another but of any central control, since it is difficult to imagine influences of these different rates arriving from the brain. Here the clock mechanism must be located in the periphery, presumably in the cells of the joints that secrete synovial fluid.

In similar manner, the distribution curve of the duration of the cycles in 38 patients with cyclic agranulocytosis (Fig. 20, top) shows an even much narrower range at 21 days. The conclusion seems to be inescapable that some mechanism or mechanisms must function in the body with this frequency. Since the chief change observed in these patients is in the number of circulating polymorphonuclear cells, we may presume that this clock is located in the tissue producing these cells. Until now, their life span has only been estimated on the basis of indirect measurements. These estimates fall as high as 13 days.[18]

The distribution curve for the cycle duration of 20 patients with Hodgkin's disease (Fig. 20, bottom) shows a wider but still definitely limited range with a

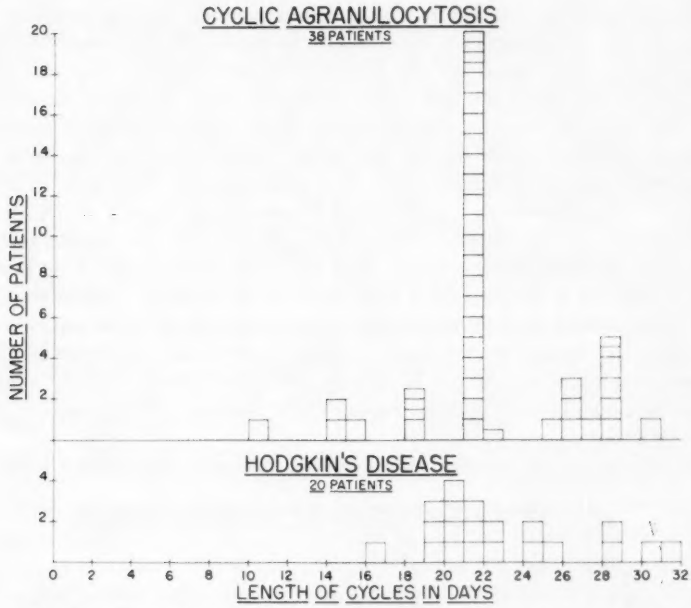

FIG. 20.—(Top) Distribution chart for 38 patients with cyclic agranulocytosis. Abscissas give lengths of cycles in days, ordinates number of patients with each length of cycle. (Bottom) Distribution chart for 20 patients with Hodgkin's disease.

peak at 20 days. Here again we are led to assume some mechanism in the body functions at this rate. The lymph glands and spleen are the organs most obviously affected in these patients; they are also the organs specifically involved in the production of lymphocytes. We would, therefore, expect to find the clock controlling Hodgkin's disease located in these tissues. Of interest here is that the life span of lymphocytes has been calculated to be 20–21 days,[19] which coincides almost exactly with the units of the postulated clocks.

Figure 21 shows the distribution curve for the cycle duration of 31 catatonic-

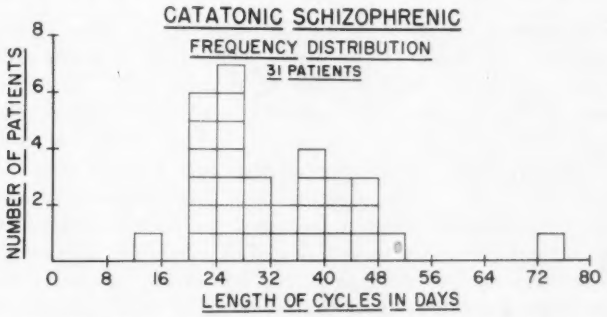

FIG. 21.—Distribution chart for 31 catatonic-schizophrenics. Abscissas show length of cycles in 4-day periods; ordinates number of patients with length of cycles falling within each 4-day period.

schizophrenic patients. As before, the abscissas give the average length of cycle for each patient. The lengths of the cycles for any one individual tended to remain quite constant. Here again we see a delimited distribution, only one patient with a cycle length below 20, and only 2 above 48 days. It seems reasonable to assume that there is a mechanism within the body which in different individuals functions at different levels within this range. The finding that most of these patients responded to administration of thyroid powder, thyroxin, or triiodothyronine[13, 20-23] suggests that a deficiency of circulating thyroid is important in the development of this illness. This is also suggested by our finding that very similar cycles of spontaneous activity, food and water intake, have been produced in rats by removal or destruction of large parts of the thyroid; further that treatment of these animals with thyroid preparation eliminated the abnormal cycles.[24, 25] Noteworthy also is that on the basis of daily observations of follicles of thyroid tissue auto-implanted to the ear of rabbits, Williams[26] reported many years ago that the individual follicles undergo regular cyclic changes. The longest cycle was found to be 20 days.

Figure 22 shows the number of psychiatric patients (108) whose cycles had

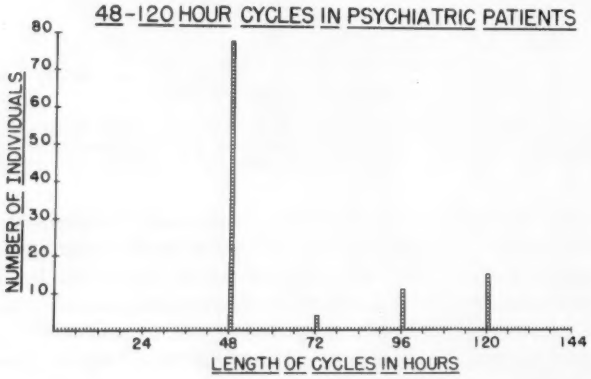

FIG. 22.—Distribution chart for 108 psychiatric patients with length of cycles of 48, 72, 96, and 120 hours respectively.

lengths falling respectively at 48 hours (78 patients), 72 hours (4 patients), 96 hours (11 patients), 120 hours (15 patients). Therefore we might expect also to find mechanisms within the body functioning at these rates. According to their histories many of these patients have suffered severe head injuries, illnesses with high fever, great stress in one form or another. They showed a variety of corresponding cyclic autonomic disturbances in pulse rate, hours of sleep, blood chemistry, appetite, thirst, etc. Thus, it seems likely that the clocks involved may be located in the hypothalamus, which is the site of the nuclei controlling these functions. Head trauma seems apt to be followed by symptoms occurring in 48-hour cycles, while lethargic encephalitis is more often followed by 120-hour cycles.[27]

It must be pointed out here again that evidence at hand indicates that an individual may display symptoms of the presence of several clocks at one time. We have already seen instances in which a patient with intermittent hydrarthrosis

harboured a clock in each of two or three or more joints, each clock measuring time in its own unit. We have also seen instances in which individuals displayed the presence of several different types of clocks—in one case an 18-day cycle of neutropenia and a 34-day cycle of menstruation. There are other instances in which individuals have shown three or more such different types of clocks, for instance a 9-day swelling of the knees, a 7-day cycle of abdominal pain, and a 14-day cycle of renal disturbances. In such patients the peaks of body temperature may not show any obvious periodicity, thus giving the impression of a noncyclic phenomenon; however, plotting of the various specific symptoms that accompany the nonspecific fever peaks often bring out clearly the presence of several independent cycles.

THREE TYPES OF BIOLOGICAL CLOCKS

In reviewing all the data collected in these patients with various kinds of cyclic disturbances in behavior, mood, and metabolism, it appears that the human body harbours at least three different types of biological clocks:

Type I: *Peripheral Clocks.*—These are located, for instance, in the synovial fluid-producing tissue in the joints, in the cell-producing tissue in bone marrow, etc. They are to a large degree independent of influences arising elsewhere in the organism, particularly in the pituitary gland and the hypothalamus. Thus, for instance, as was mentioned above, cyclic swelling of the joints is generally the only pathological change demonstrable in patients with intermittent hydrarthrosis. There is no fever, no increase in pulse rate, no leucocytosis. Pain may be the only accompanying symptoms. But that these clocks are not completely independent of the rest of the organism is shown by the fact that most of them can be temporarily stopped by pregnancy. However, because of their high degree of independence of other parts of the organism, these clocks are very accurate.

Type II: *Central Clocks.*—These clocks are located in the central nervous system. On the basis of our present knowledge we assume they are most likely to be found in the thalamus, hypothalamus, reticular formation, or in the posterior lobe of the pituitary gland.

I have included the posterior lobe of the pituitary gland as a possible harbourer of a clock, or clocks, of this type, since it actually is part of the brain, and since in animal experiments damage to this structure brought out extraordinarily regular but abnormal cycles in running activity, food and water intake, and functioning of the reproductive tract.[28] From results of our own animal experiments we can conclude that the region of the infundibulum contains more cyclic mechanisms than any other part of the brain or body. But just which structures in this intricate region are the actual sites of such clock mechanisms is not known. Nor do we know to what extent they are controlled through neuro-hormonal secretion in the hypothalamus. At least some of these central clocks keep time with a high degree of accuracy and, again, independently to a large extent of influence from other parts of the body. Thus, as an instance, in one patient a 48-hour clock operated without a miss for over 30 years, despite all the many influences, external and internal, to which he must have been exposed during this time.[29]

Many observations reported in the literature on animals, insects, and plants have established the high degree of accuracy of the functioning of the 24-hour clock, the chief representative of this type of clock.[30–34]

Type III: *Homeostatic Clocks.*—These are part of the homeostatic mechanisms that include the interaction between the target glands of internal secretion—the thyroid, parathyroids, gonads, adrenals, and the anterior lobe of the pituitary gland and/or the hypothalamus. An example would be the ovarian or menstrual clock that depends on the homeostatic relationship between a target endocrine gland, the ovary, and the pituitary and hypothalamus. Another example would be the presumably thyroid clock of catatonic-schizophrenics which would be regulated by the homeostatic mechanism including the thyroid, pituitary, and hypothalamus.

Compared to the Type I (peripheral) clocks and to Type II (central) clocks, these are much less accurate and operate over a much wider range of variation. Thus, for example, the ovarian or menstrual clock measures time most frequently in units of 27–28 days but the length of the units ranges from 14 to 100 days, as may be seen in the distribution chart of the characteristic duration of the menstrual cycles of 1,165 "normal" women (Fig. 23).[35] The ordinates give the number of

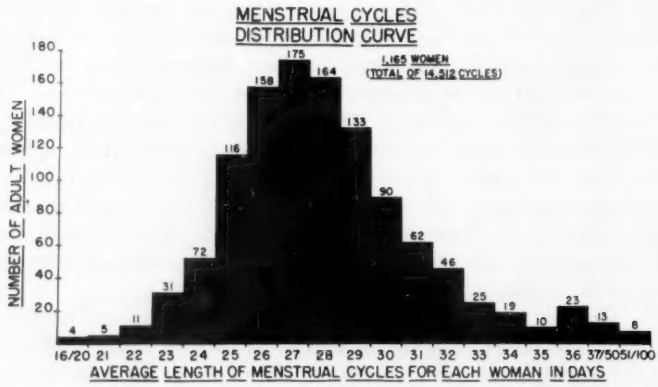

FIG. 23.—Distribution chart of average lengths of menstrual cycles for 1,165 females—a total of 14,512 cycles. (Redrawn from Arey, 1939.)

women with characteristic cycles of each duration (indicated on the abscissas). Similar curves have been obtained by others, both in human beings and monkeys.[36, 37]

In these clocks the actual timing mechanisms are probably located in the target glands but they are very definitely under the influence of secretion from the anterior lobe of the pituitary, as well as being controlled by specific centers in the hypothalamus and thalamus. It is possible that impulses from the sympathetic and the parasympathetic components of the autonomic nervous system may also play a part in the regeneration of this clock mechanism.

Conditions Under Which These Three Types of Clocks Are Revealed.—Shock, trauma, and allergy[38] are probably the most common forerunners of the appearance of Type I and Type II clocks. Prolonged spells of fever, prolonged treatment with any one of a variety of drugs or hormones, and presence of arteriosclerosis or of lues have been reported as antecedents of these two types of clocks. However, instances are also on record in which these clocks made their appearance apparently spontaneously.

Particular susceptibility to cyclic manifestations may also play a part since instances are known in which individuals—even within the first few months of life—show evidence of harbouring not only one but several clocks, each measuring time in quite different units, and all independent of one another. This is true especially of the large groups of patients who from early ages suffer from the so-called Mediterranean disease, otherwise known as paroxysmal peritonitis, or periodic abdominalgia.[39] Evidence at hand indicates that this particular susceptibility has a definitely genetic origin, as Armenians and Jews show a high incidence of cyclic proneness and also because, in a number of instances, abnormal cyclic phenomena have appeared in as many as five generations of a family.[40]

In contrast to the other two types of clocks, the usual condition for the appearance of Type III clocks must be either a great reduction of functional activity in the target gland or of the cells of the anterior pituitary that secrete the corresponding trophic hormone. Another possibility which can be anticipated to result in the manifestation of this Type III clock would be consequent upon functional or structural changes in hypothalamic centers resulting from prolonged attempts of the organism to maintain homeostasis in the face of a great reduction in amounts of functioning tissue of the target glands.[24] The results of our studies in experimental production of abnormal cycles of behavior and metabolism in rats indicate that loss of function of the frontal lobes and presumably of the resultant loss of inhibitory influences on lower centers may be followed by the appearance of Type III clocks.[25] The results of these studies on rats have likewise shown that conditions of general debilitation are conducive to the appearance of abnormal cycles.

TREATMENT OF INDIVIDUALS WITH CYCLIC MANIFESTATIONS OF ILLNESS

It was hoped that light might be thrown on the operation of the clock mechanisms by the study of methods of treatment that have resulted in disappearance of abnormal cycles. However, this study did not reveal much useful information since only a very few effective treatments for any of the cyclic conditions are known. Up to the present time no consistent treatment has been found for patients showing any of the Type I (peripheral) clocks, but a great variety of treatments at different times in one patient or another have resulted in the loss of all signs of the presence of a clock. Thus, for example, in the case of patients suffering from intermittent hydrarthrosis, temporary or permanent cures have been achieved by the following: cauterization of synovial membranes, drainage of the synovial spaces, puncturing or washing out of these spaces with mercuric chloride or carbolic acid, injection of iodine into the synovial spaces, or the oral administration of drugs such as arsenic and quinine.

Many patients showing Type II (central) clocks have made spontaneous recoveries, but to my knowledge none has been helped by any form of treatment.

Symptoms evidencing the presence of Type III (homeostatic) clocks have responded to treatment with thyroid hormone, extract of thyroid, thyroxin, or triiodothyronine. One of our patients suffering from parathyroid deficiency and showing very regular 40-day cycles in mood and behavior lost all signs of periodic changes after treatment with AT-10 and oral ingestion of large amounts of solutions of calcium lactate.

PATHOLOGICAL FINDINGS IN PATIENTS MANIFESTING CLOCKS

As stated earlier, remarkably few pathological changes have been demonstrated in any patients revealing the presence of biological clocks of any type. Thus, in patients with intermittent hydrarthrosis many examinations of synovial fluid or membranes have yielded very few if any evidences of pathological changes.

The many laparotomies performed in patients with paroxysmal peritonitis have revealed the presence of peritoneal inflammation, but nothing more; inflammation disappeared at the end of each attack.

Examination of the brains of patients with cyclic disorders similarly have not revealed any pathological changes—with the exception of patients with histories of earlier specific central nervous system disorders, such as lethargic encephalitis, progressive paralysis, etc.

EVOLUTION OF CLOCKS

Evidence at hand indicates that the 24-hour clock—which is found not only in man but in other animals, birds, insects, and plants—is the most primitive clock. How and when the other clocks appeared in different animals, what significance and selective value they had in the life of the animals, remains unknown. It would appear that cyclic phenomena are more readily visible in lower animals than in man. It is possible that, in the process of evolution, clocks that once may have played a part in the animal's life are gradually becoming concealed until in many only two clocks, the 24-hour and 28-day menstrual clocks, are still visible, and that they too may ultimately become concealed, to make their appearance only under various forms of pathological conditions.

In summary, thus far, the results of this study have shown that the body harbours a number of clocks—some located in peripheral organs, for instance in the joints; some located in endocrine glands, for instance the thyroid; some located in the nuclei of the hypothalamus and near-by parts of the brain. It may now be asked how these clocks work; how the various organs of the body undergo such marked periodic changes over long periods of time without showing signs of damage—either on gross or histological examination; how shock, trauma, or other agents elicit cyclic responses; how an assortment of agents may cause the cyclic responses to disappear?

SHOCK-PHASE HYPOTHESIS

From the prolonged consideration of these questions an idea emerged that may provide an answer to at least some of these questions. This idea came partly from the detailed study of the case histories of the large variety of patients possessing clocks, especially those seen in patients with intermittent hydrarthosis, and partly from the study of a phenomenon that has been observed in the common fruit fly.[41–45] It has been reported that the pupa of the common fruit fly has an inherent 24-hour emergence rhythm; further, that when a large number of pupae are kept together in the absence of any outside stimuli, that is, in constant darkness, at a constant temperature, etc., the flies emerge at all times of the day and night, giving a fairly constant emergence rate until all pupae have emerged; it has further been reported that a shock, short flash of light or thermal stimulus, may serve to bring all of the pupae into phase so that they emerge at 24-hour intervals

until all have pupated. It occurred to me that what happens to the individual members of a colony of flies in response to a strong shock might also happen to the individual units of an organ that are physically closely bound together into an integrated whole. This idea has taken shape as the "shock-phase" hypothesis.

This hypothesis consists of the following postulates:

1. That each one of the functioning units of the organism—cells, follicles, glomeruli, neurons, etc.—has an inherent cycle, the length of which is characteristic of that organ.

2. That these units, although physically closely bound together, may operate independently of one another. Thus, they may all be active, or inactive, or at various stages between activity and rest.

3. That in a normal healthy organ these units function out of phase, that is, some are active while others are resting or at various intermediate stages, thus insuring a fairly constant level of productivity.

4. That under certain circumstances these randomly functioning units of an organ may all be synchronized by a shock or trauma, or by other forms of interference, thus revealing the lengths of the inherent cycles of the individual units.

5. That under certain circumstances the synchronized units of an organ may be desynchronized by various forms of interference, thus restoring the organ to its normal noncyclic productivity.

According to this hypothesis, for instance, the fluid-producing organs of the joints are composed of individual units, each one having its own inherent cycle, ranging in length from 7–14 days. For most individuals the lengths of the cycles of the units are the same in all joints; in other individuals the units within different joints have cycles of different lengths. Under normal conditions some of the units are active, others are resting, others in between, thus maintaining production of a fairly constant amount of synovial fluid from day to day and so concealing all signs of the presence of cyclic phenomena in the individual units. After local injury, allergic shock, etc., the units are brought into phase to produce the great swelling of the joints at 7–14 day intervals. Various forms of nonspecific treatment as was pointed out above, may then desynchronize the units to return the joints to their normal condition.

Further, according to this hypothesis, in normal individuals the neutrophil-producing units in the bone marrow, likewise, have their inherent rhythm of 21, or nearly 21, days, and are capable of independent functioning. Under normal conditions some of these units are producing neutrophils, others are resting, others are at various stages between. Under abnormal conditions these units may all be brought into phase, so that at one time all functioning units are producing cells, at another, none.

According to this theory the same principles apply to: the lymph-producing units in the lymph glands or spleen; the hormone-controlling neurons of the various nuclei in the brain stem; likewise the acid-producing cells of the gastric mucosa; or to the fluid-producing cells of the peritoneal membranes in the abdominal and thoracic cavities, etc.

Evidence for Hypothesis.—At the present time we know of a number of organs the functioning units of which may under normal conditions be in all stages of activity, some very active, some completely inactive, the others at various stages between.

It is a common observation that the follicles of the thyroid in a normal animal or person are at any one time apt to be in all stages of activity and rest, and that under pathological conditions they may likewise all be in a state of hyperactivity, or in a state of complete inactivity. In his studies on follicles of autotransplants of thyroid tissue, Williams found that individual follicles pass through definite cycles of growth—and quite independently of one another.[26]

It is known on the basis of studies of the activity of the cells of the gastric mucosa made by means of special dyes that these cells at any one time may be at all stages of activity and inactivity.[46]

Further, it is known that the uterus of normal females shows all stages of activity and inactivity and independence of the rest of the organism.[47]

Some evidence at hand indicates that the glomeruli of the kidneys may likewise function quite independently of one another.[48] However, we have no idea whether the glomeruli have an inherent cycle, and if so of what length.

Evidence is also at hand indicating that the individual neurons of the various centers of the brain may function independently of one another.[49]

SUMMARY

1. Human beings harbour many clocks—far more than hitherto has been suspected, most of which manifest their presence only under pathological conditions.

2. These clocks may manifest their presence either through physical signs or symptoms, involving in one individual or another almost every organ of the body, or in primarily mental and emotional symptoms, involving almost every form of abnormal behavior, or mood, and thinking.

3. The clocks show an apparent degree of independence of all external physical influences—temperature, humidity, barometric pressure, etc.—as well as of day-to-day emotional situations or disturbances, and so depend on inherent mechanisms.

4. These various clocks keep time in units that range in length from 12 hours to several years; but for each clock the units tend to remain quite constant.

5. Our records indicate the existence of three types of clocks:

I. Peripheral—those located for instance in the blood-forming tissues in the bone marrow, etc.

II. Central—those located in the brain, particularly in the hypothalamus and reticular formation.

III. Homeostatic—those mechanisms involving target organs, endocrine glands, the pituitary, and hypothalamus.

6. A "shock-phase" hypothesis in agreement with the observed clinical phenomena has been proposed to explain how the clocks work. It is assumed that the functioning units of every organ of the body—cells, follicles, neurons, glomeruli, etc.—have an inherent cycle, characteristic of the organ. Under normal conditions these units of any given tissue function out of phase; under abnormal conditions they may function in phase, thus disclosing the length of the inherent cycle of the organ. The units may be put in phase by shock, trauma, allergy, etc.; similarly, they may be put out of phase again by a variety of agents and forms of treatment.

7. Patients with cyclic phenomena offer an excellent opportunity for the

study of the physiology of individual organs as well as of the functioning of the total organism, particularly as seen in various abnormal mental and emotional conditions.

* This material was presented in part in the Thomas Salmon Lectures delivered in New York City, December 2, 1959. The published lectures give a full account of our observations of biological clocks not only of man but of animals; much of the present material was presented in a symposium on "Biological Clocks" organized by the Long Island Biological Laboratories and held at Cold Spring Harbor in June, 1960. Drs. E. A. Park and K. K. Rice made many helpful criticisms and suggestions.

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